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Procedure to Block Rho-Kinase (ROK) Activation and Breast
Tumor Metastasis

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13. ABSTRACT (Maximum 200 Words) In this study we have examined CD44 [an hyaluronan (HA) receptor] interaction with a RhoA-specific guanine nucleotide exchange factor (p115RhoGEF) in human metastatic breast tumor cells (MDA-MB-231 cell line). Immunoprecipitation and immunoblot analyses indicate that both CD44 and p115RhoGEF are expressed in MDA-MB-231 cells, and that these two proteins are physically associated as a complex <i>in vivo</i> . The binding of HA to MDA-MB-231 cells stimulates p115RhoGEF-mediated RhoA signaling and Rho-Kinase (ROK) activity which, in turn, increases serine/threonine phosphorylation of the adaptor protein, Gab-1 (Grb2-associated binder-1). Phosphorylated Gab-1 promotes PI3 kinase recruitment to CD44v3. Subsequently, PI3 kinase is activated (in particular, α , β , γ forms but not the δ form of the p110 catalytic subunit), AKT signaling occurs, the cytokine [macrophage-colony stimulating factor (M-CSF)] is produced and tumor cell-specific phenotypes (e.g. tumor cell growth, survival and invasion) are upregulated. Furthermore, we have found that overexpression of a dominant-negative form of ROK [by transfection of MBA-MD-231 cells with ROK's Rho-binding (RB) domain cDNA] not only inhibits HA/CD44-mediated RhoA-ROK activation and Gab-1 phosphorylation, but also downregulates oncogenic signaling events (e.g. Gab-1/PI3 kinase-CD44v3 association, PI3 kinase-mediated AKT activation and M-CSF production) and tumor cell behaviors (e.g. cell growth, survival and invasion). Taken together, these findings strongly suggest that CD44 interaction with p115RhoGEF and ROK plays a pivotal role in promoting Gab-1 phosphorylation leading to Gab-1-PI3 kinase membrane localization, AKT signaling and cytokine (M-CSF) production during HA-mediated breast cancer progression.				
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INTRODUCTION

CD44 [an hyaluronan (HA) receptor] belongs to a family of multifunctional transmembrane glycoproteins expressed in a number of tissues and cells including breast tissues and cells (1-6). It is encoded by a single gene which contains 19 exons (7). Of the 19 exons, 12 exons can be alternatively spliced (7). Most often, breast tumor cells and tissues express several different CD44 spliced variant (CD44v) isoforms in addition to CD44s (the standard form) and CD44E (the epithelial form) (7). Because various CD44 isoforms mediate different functions, much attention has been paid to the changes in CD44v isoform production during malignant transformation (1-6). Specifically, there is accumulating evidence that the induction and overexpression of CD44v isoforms are associated with the invasion and progression of breast carcinomas and tumor cell lines (1-6). These CD44v isoforms appear to confer the malignant properties of abnormal adhesion, growth, migration and invasion (1-6). Furthermore, CD44 has been shown to interact with extracellular matrix components, (ECM) [e.g. hyaluronan (HA) at the N-terminus of the extracellular domain] (8-10) and to contain specific binding sites for the cytoskeletal proteins (e.g. ankyrin and ERM) within the 70 amino acid C-terminus of its cytoplasmic domain (11-16).

Several mechanisms for the regulation of HA/CD44-mediated function have been suggested. These include modifications by an additional exon-coded structure (via an alternative splicing process) (1-6), variable N-/O-linked glycosylation on the CD44's extracellular domain (17,18); and selective interactions of the CD44's cytoplasmic domain with ankyrin (11,15) and various signaling molecules [e.g. the Src family tyrosine kinases (14,19), p185^{HER2} (20,21), Rho-Kinase (ROK) (22), TGF- β receptor kinases (23), and the guanine nucleotide exchange factors, Tiam1 (24) and Vav2 (21)]. In addition, CD44 has been shown to be involved in the production of cytokines [e.g. interleukin 8 (IL-8) (1) and FGF-2 (13)] and hormones [e.g. parathyroid hormone-related protein (PTHrP) (23)] in breast tumor cells. These findings clearly indicate that CD44 plays a pivotal role in activating oncogenic signaling and HA-mediated breast tumor cell function.

Members of the Rho subclass of the Ras superfamily [small molecular weight GTPases, (e.g. RhoA, Rac1 and Cdc42)] are known to transduce signals regulating many cellular processes (25). The rationale for our focusing on RhoGTPase is based on previous reports suggesting that CD44-associated cytoskeletal proteins (e.g. ankyrin and ERM) and tumor cell-specific phenotypes are dependent on RhoGTPase signaling events (22,26,27). Overexpression of certain RhoGTPases in human tumors often correlates with poor prognosis (28,29). In particular, coordinated RhoGTPase signaling is considered to be a possible mechanism underlying cell proliferation and motility, an obvious prerequisite for metastasis (25-31). Presently, very little information is available regarding how they are activated by cell surface receptors. To date, at least 30 different guanine nucleotide exchange factors (GEFs) have been identified (32). Our recent results indicate that the interaction between CD44v3 isoform and two guanine nucleotide exchange factors (GEFs), such as Tiam1 (24) and Vav2 (21), upregulates Rac1 signaling and cytoskeleton-mediated metastatic tumor progression. As part of our continued effort to identify CD44 isoform-linked GEFs which correlate with certain metastatic behaviors, a new candidate molecule, named p115RhoGEF, has been identified. p115RhoGEF, which is the human homolog of the mouse protein Lsc, is involved in RhoA activation (33); and structurally, it contains numerous functional domains and structural motifs found

in signal transduction proteins and oncoproteins. These motifs include amino-terminal RGS (Regulator of G protein Signaling) domain, a dbl homology domain (DH), and a pleckstrin homology domain (PH)(33,34). In particular, the sequence between aa421-aa635 contains significant sequence homology to the Dbl-homologous domain (DH) of many proteins which exhibit GDP/GTP exchange activity for specific members of the Ras superfamily of GTP-binding proteins (32). The p115RhoGEF has been clearly shown to act as a GDP/GTP exchange protein for the Rho subfamily of GTPases, including RhoA (33). In addition, p115RhoGEF (similar to PDZ-RhoGEF) contains an RGS domain which interacts with Gα13 and regulates the ability of DH to carry out GDP/GTP exchange activity for RhoA (33,34). In the carboxyl-terminal region, p115RhoGEF contains one pleckstrin homology domain (PH) which is commonly detected in signaling molecules and cytoskeletal proteins (35,36). Activation of RhoGTPases such as RhoA have been shown to produce specific structural changes in the plasma membrane associated with actin filament bundling, stress fiber formation and acto-myosin-based cytoskeletal function (25).

Several different enzymes have been identified as possible downstream targets for RhoA signaling. One such enzyme is Rho-Kinase (ROK-also called Rho-associated kinase) which is a serine-threonine kinase known to interact with Rho in a GTP-dependent manner (37-39). ROK is composed of four functional domains including a kinase domain (catalytic site), a coiled-coil domain, a Rho-binding (RB) domain and a pleckstrin-homology (PH) domain (40). This enzyme has been shown to regulate cytoskeleton function by phosphorylating several important cytoskeletal regulators including myosin-binding subunit (MBS) of myosin phosphatase (41), calponin (42), adducin (43) and LIM kinase (44). ROK is also involved in the “cross-talk” between Ras and Rho signaling leading to cellular transformation (30). We have demonstrated that ROK phosphorylates the cytoplasmic domain of CD44_{v3,8-10} isoform and up-regulates the interaction between CD44_{v3,8-10} isoform and the cytoskeletal protein, ankyrin, during HA/CD44-regulated tumor cell migration (22). Most recently, the pleckstrin-homology (PH) domain of ROK is found to be involved in the direct binding to CD44 and HA-mediated Ca²⁺ signaling in endothelial cell function (45). Thus, ROK is clearly one of the important signaling molecules required for membrane-cytoskeleton interaction, Ca²⁺ regulation and HA/CD44-mediated cell function (22,45).

In the pathogenesis of cancer, Gab-1 (Grb-2-associated binder-1) and phosphatidylinositol 3-kinase (PI3 kinase) are key mediators in regulating oncogenesis [for review see (46)]. Specifically, Gab-1 [a member of the insulin receptor substrate (IRS) family] functions as one of the major adapter molecules downstream of growth factor signaling (47,48). Gab-1 also possesses multiple phosphorylation sites that could act as docking sites for PI3 kinase known to consist of a catalytic subunit p110 (α, β, and δ) and regulatory subunit p85 (α, β, and p55γ) or the catalytic subunit p110γ and the regulatory subunit p101 [for reviews see (46,49-51)]. One recent study found a positive link between HA-CD44 interaction and Gab-1-associated PI3 kinase activation during the stimulation of cellular transformation by a Met-hepatocyte growth factor oncoprotein (Tpr-Met) (52). HA and CD44 also promote PI3 kinase signaling in a tumor cell-specific manner. For example, PI3 kinase participates in CD44-mediated survival pathway in colon carcinoma cells (53). HA activates PI 3 kinase-AKT pathways leading to cell motility and cell survival-signaling pathways (54). The active mutant of p110 subunit of PI3 kinase exerts its action on the cleavage of CD44 during cancer cell migration (55). These findings suggest PI3 kinase activation is closely coupled with HA-mediated

CD44 signaling.

Because both Rho signaling and PI3 kinase activation play an important role in regulating breast tumor progression, we have focused in this study on the relationship between these two signaling pathways during HA/CD44-mediated breast tumor progression. A unique mechanism is described concerning CD44 interaction with p115RhoGEF and ROK that stimulates Gab-1 phosphorylation/membrane localization and PI3 kinase-AKT activation leading to HA/CD44-regulated cytokine production and tumor cell behaviors required for breast cancer progression.

BODY

MATERIALS AND METHODS

Cell Culture: The breast tumor cell line (MDA-MB-231 cells) was obtained from the American Type Culture Collection (ATCC) and grown in Eagle's minimum essential medium (EMEM) supplemented with Earle's salt solution, essential and non-essential amino acids, vitamins and 10% fetal bovine serum.

Antibodies and Reagents: Monoclonal rat anti-human CD44 antibody (Clone:020; Isotype: IgG_{2b}; obtained from CMB-TECH, Inc., San Francisco, CA.) used in this study recognizes a common determinant of the CD44 class of glycoproteins. Both rabbit anti-CD44v3 antibody and rabbit anti-Rho-Kinase (ROK) were prepared according to the procedures described previously (22). For the preparation of polyclonal rabbit anti-p115RhoGEF antibody, specific synthetic peptides [~15-17 amino acids unique for the ROK or p115RhoGEF sequence] were prepared by the Peptide Laboratories using an Advanced Chemtech automatic synthesizer (model ACT350). All polyclonal antibodies were prepared using conventional DEAE-cellulose chromatography and tested to be monospecific (by immunoblot assays). Mouse monoclonal anti-green fluorescent protein (GFP) and mouse monoclonal anti-FLAG (M2) were purchased from PharMingen and Sigma, respectively. Rabbit anti-phospho-threonine antibody and rabbit anti-phospho-serine antibody were obtained from Zymed Laboratories Inc. Monoclonal mouse anti-p110 α , mouse anti-p110 β , mouse anti-p110 γ and mouse anti-p110 δ were purchased from Santa Cruz Biotechnology. Several other immuno-reagents including mouse anti-AKT-1 (PKB), rabbit anti-phospho-AKT-1 (Threonine³⁰⁸) and rabbit anti-Gab-1 were purchased from Upstate Biotechnology, Inc. The specific inhibitor of PI3 kinase (LY294002) was obtained from Calbiochem. Rooster comb hyaluronan (HA) was purchased from Sigma. High molecular weight HA polymers (~10⁶ daltons) were purified by gel filtration column chromatography using Sephacryl S1000 column as described previously (18). The purity of high molecular weight HA polymers used in our experiments was further verified by anion exchange high-performance liquid chromatography (HPLC). No small HA fragments was detected in these preparations.

Cloning, Expression and Purification of CD44 Cytoplasmic Domain (CD44cyt) from E. coli: The cytoplasmic domain of human CD44 (CD44cyt) was cloned into pFLAG-AST using the PCR-based cloning strategy (15). Using human CD44 cDNA as template, one PCR primer pair (left, FLAG-EcoRI; right, FLAG-XbaI) was designed to amplify complete CD44 cytoplasmic domain.

The amplified DNA fragments were one-step cloned into a pCR2.1 vector and sequenced. Then, the DNA fragments were cut out by double digestion with EcoRI and XbaI and subcloned into EcoRI/XbaI double-digested pFLAG-AST (Eastman Kodak Co.-IBI, Rochester, NY) to generate FLAG-pCD44cyt construct. The nucleotide sequence of FLAG/CD44cyt junction was confirmed by sequencing. The recombinant plasmids were transformed to BL21-DE3 to produce FLAG-CD44cyt fusion protein. The FLAG-CD44cyt fusion protein was further purified by anti-FLAG M2 affinity gel column (Eastman Kodak Co.-IBI, Rochester, NY). The nucleotide sequence of primers used in this cloning protocol is: FLAG-EcoRI: 5'-GAGAATTCGAACAGTCGAAGAAGGTGTCTCTTAAGC-3'; FLAG-XbaI: 5'-AGCTCTAGATTACACCCCAATCTTCAT-3'.

Cloning and Expression of the PH Domain of Gab-1: The Gab-1cDNA fragment containing the PH domain (bp 132 – 482; Accession: NM_002039) was amplified by RT-PCR using PH-specific primers (upper primer containing a start codon) 5'-GCCATGGGTGAAGTGGTCTGCTCCGGATG-3' and 5'-TGTTGGATTAAACCCACAGATGTCACA-3'. The Gab-1-PHcDNA fragment was cloned into pcDNA3.1-V5/His TOPO vector (Invitrogen). The inserted PH-sequence was confirmed by nucleotide sequencing analyses (UCSF Biomolecular Resource Center). This V5/His-tagged Gab-1-PH fragment cDNA was then used for transient expression in COS-7 cells or MDA-MB-231 cells as described below. The expression of His-tagged Gab-1-PH domain in COS-7 or MDA-MB-231 cells was detected by SDS-PAGE and immunoblot/immunoprecipitation analyses.

In Vitro Binding of CD44cyt to Gab-1's PH Fragment and Intact Gab-1: Aliquots (0.5-1 ng protein) of V5/His-tagged Gab-1 PH fragment (isolated from COS-7 cells)-conjugated Sepharose beads or V5/His-conjugated beads were incubated in 0.5 ml of binding buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% bovine serum albumin and 0.05% Triton X-100] containing FLAG-CD44cyt fusion protein (as described above) at 4°C for 4 h. The binding equilibrium was found to be established when the *in vitro* CD44-Gab-1-PH binding assay was conducted at 4°C after 4 h. Following binding, the Gab-1-PH-V5/His-conjugated beads or V5/His-conjugated beads were washed extensively in binding buffer followed by immunoblotting with anti-FLAG antibody. In some cases, ¹²⁵I-labeled CD44cyt (the cytoplasmic domain of CD44 fusion protein) (~0.35nM protein, 2 x 10⁴ cpm/ng) was incubated with Sepharose-beads containing intact Gab-1 phosphorylated by ROK plus activated RhoA [RhoA with GTPγS bound (GTPγS•RhoA)] or ROK plus unactivated RhoA (e.g. RhoA without GTPγS bound) in the presence of various concentrations of unlabeled CD44cyt ranging from 10⁻¹² to 10⁻⁶M. The radioactivity associated with Gab-1-beads was analyzed by liquid scintillation counting.

Method for Preparing GFP (Green Fluorescent Protein)-Tagged Dominant-Negative Form [Containing Rho-Binding Domain (RB)] of Rho-Kinase (ROK): The cDNA fragment encoding the dominant-negative form of ROK (2719-3237bp, containing the Rho-binding sequence (RB)) was amplified by RT-PCR using RB-specific primers linked with enzyme (Xho I and Hind III) digestion site, 5'-CGATCTCGAGGGCCTTCTGGAGGAGAGTA-3' and 5'-CGATAAGCTTCTGCATCTGAAGCTCATTC-3'. PCR product digested with Xho I and Hind III was purified with QIAquick PCR purification Kit (Qiagen) (22). The ROK-RBcDNA fragments were cloned into

pEGFPC1 vector (Clontech) digested with Xho I and Hind III. The inserted RB sequence was confirmed by nucleotide sequencing analyses. This GFP-ROK-RBcDNA was then used for a transient expression in MDA-MB-231 cells. The GFP-tagged ROK-RB (M.W. ~50kDa) expressed in MDA-MB-231 cells was analyzed by SDS-PAGE and immunoblot as described below.

Cell Transfection: To establish a transient expression system, MDA-MB-231 cells were transfected with various plasmid DNAs (e.g. His-tagged Gab-1-PHcDNA or GFP-tagged ROK-RBcDNA or vector alone) using Lipofectamine 2000. Transfected cells were then grown in the culture medium containing G418 for at least 3 days. Various transfectants were analyzed for their protein expression (e.g. Gab-1-PH domain or ROK-RB domain) by immunoblot and functional assays as described below. In some experiments, COS-7 cells were transfected with His-tagged Gab-1 fragment cDNA (or vector alone) using Lipofectamine 2000. The expression of His-tagged Gab-1 PH domain in COS-7 was also detected by SDS-PAGE and anti-His-mediated immunoblot/immunoprecipitation analyses.

Immunoblotting and Immunoprecipitation Techniques: MDA-MB-231 cells were solubilized in 50mM HEPES (pH 7.5), 150mM NaCl, 20mM MgCl₂, 1.0% Nonidet P-40 (NP-40), 0.2mM Na₃VO₄, 0.2mM phenylmethylsulfonyl fluoride, 10μg/ml leupeptin, and 5μg/ml aprotinin. The sample was centrifuged at 14,927x g for 15 min and the supernatant was analyzed by SDS-PAGE in a 5% or 7.5% polyacrylamide gel. Separated polypeptides were then transferred onto nitrocellulose filters. After blocking non-specific sites with 2% bovine serum albumin, the nitrocellulose filters were incubated with each of the specific immuno-reagents [e.g. anti-CD44v3 IgG (5μg/ml), anti-p115RhoGEF IgG (5μg/ml), anti-ROK IgG (5μg/ml), anti-Gab-1 IgG (5μg/ml), and anti-AKT-1 IgG (5μg/ml)] followed by incubating with horseradish peroxidase (HRP)-labeled goat anti-rat IgG, or HRP-labeled goat anti-rabbit IgG. The blots were then developed by the ECL™ system (Amersham Co.). MDA-MB-231 cells were also solubilized by 1.0% NP-40 and immunoprecipitated with rabbit anti-CD44v3 antibody followed by anti-p115RhoGEF-mediated or anti-p115RhoGEF-free serum (anti-p115RhoGEF antibody pre-absorbed by an excess amount of p115RhoGEF)-mediated immunoblot; or immunoprecipitated with anti-p115RhoGEF antibody followed by anti-CD44v3-mediated or anti-CD44v3-free serum (anti-CD44v3 antibody pre-absorbed by an excess amount of CD44v3)-mediated immunoblot, respectively.

For analyzing the complex formation between Gab-1/PI3 kinase (e.g. p110α, p110β, p110γ or p110δ form) into CD44v3 complex, MDA-MB-231 cells [untransfected or transfected with His-tagged Gab-1-PHcDNA or GFP-tagged ROK-RBcDNA or vector alone] treated with various reagents [e.g. HA (50μg/ml; Sigma Chemical Co.) or pre-treated with anti-CD44 antibody followed by HA (50μg/ml) or without HA treatment] were solubilized by 1.0% NP-40 and immunoprecipitated with rat anti-CD44 antibody followed by immunoblotting with various immuno-reagents [e.g. anti-His, anti-Gab-1 or anti-PI3 kinase antibodies (e.g. anti-p110α, anti-p110β, anti-p110γ or anti-p110δ)]. In some experiments, NP-40 solubilized cell lysate isolated from MDA-MB-231 cells [untransfected (treated with or without LY294002) or transfected with GFP-tagged ROK-RBcDNA or vector alone-treated with HA (50μg/ml) or pre-treated with anti-CD44 antibody followed by HA (50μg/ml) or without HA treatment] was analyzed by SDS-PAGE followed by immunoblotting with anti-GFP or anti-phospho-AKT-1 (50μg/ml) followed by re-blotting with anti-

AKT-1 (50µg/ml). These blots were then treated with peroxidase-conjugated goat anti-rabbit IgG and ECL chemiluminescence reagent.

Some MDA-MB-231 cells [untransfected (treated with or without LY294002) or transfected with ROK-RBcDNA or vector alone-treated with HA (50µg/ml) or pre-treated with anti-CD44 antibody followed by HA (50µg/ml) or without HA treatment] were also immunoprecipitated with anti-Gab-1 antibody followed by immunoblotting with anti-phospho-serine or anti-phospho-threonine, respectively. These blots were then developed using ECL chemiluminescence reagent according to the manufacturers instructions. During these immunological analyses, an equal amount of cellular protein (50µg/ml) immunoprecipitated with the antibody was applied to SDS-PAGE followed by immunoblot analyses.

p115-Mediated GDP/GTP Exchange For RhoA: Purified E. coli-derived GST-tagged RhoA or GST alone (20pmole) was preloaded with GDP (30µM) in 10µl buffer containing 25mM Tris-HCl (pH 8.0), 1mM DTT, 4.7mM EDTA, 0.16mM MgCl₂ and 200µg/ml BSA at 37° for 7min. In order to terminate preloading procedures, additional MgCl₂ was then added to the solution (reaching a final concentration of 9.16mM) as described previously (16,17,59). Subsequently, 2 pmole of p115RhoGEF [bound to anti-p115RhoGEF-conjugated Sepahrose beads] isolated from MDA-MB-231 cells grown in the presence or absence of HA (50µg/ml) was preincubated with 2.5µM [³⁵S]GTPγS (≈1,250Ci/mmol) (in the presence or absence of 2.25µM GTPγS) for 10 min followed by adding 2.5pmole GDP-loaded GST-tagged RhoA (or GDP-treated GST). At various time points, the reaction of each samples was terminated by adding ice-cold termination buffer containing 20mM Tris-HCl (pH 8.0), 100mM NaCl, and 10mM MgCl₂ followed by filtering through nitrocellulose filters. The radioactivity associated with the filters was measured by scintillation fluid. The amount of [³⁵S]GTPγS bound to p115RhoGEF (bound to anti-p115RhoGEF-Sepharose beads) or control sample (preimmune serum-conjugated Sepharose beads) in the absence of GST-tagged RhoA was subtracted from the original values. In some cases, p115RhoGEF-catalyzed GDT/GTP exchange reactions were measured using MDA-MB-231 cells pre-incubated with monoclonal rat anti-CD44 antibody (or normal rat IgG) followed by HA treatment or fibronectin treatment (or no treatment). Data represent an average of triplicates from 3-5 experiments. The standard deviation was less than 5%.

Protein Phosphorylation Assay In Vitro: The kinase reaction was carried out in 50 µl of the reaction mixture containing 40 mM Tris-HCl (pH7.5), 2 mM EDTA, 1 mM DTT, 7 mM MgCl₂, 0.1% CHAPS, 0.1 µM calyculin A, 100 µM ATP, purified enzymes (e.g. 100 ng MDA-MB-231 cells' ROK or no ROK) and 1 µg Gab-1 (obtained from anti-Gab-1-associated beads) in the presence or absence of GTPγS-GST-RhoA fusion protein (1 µM) or GST-RhoA alone (1 µM). After an incubation for various time intervals (e.g. 0, 10, 20, 30, 60 and 120 min) at 30°C, the reaction mixtures were boiled in SDS-sample buffer and subjected to SDS-PAGE. The protein bands were revealed by anti-phospho-serine/anti-phospho-threonine-mediated immunoblot and anti-Gab-1-mediated immunoblot, respectively.

Measurement of PI3 Kinase Activity: MDA-MB-231 cells [untransfected (treated with or without LY294002) or transfected with ROK-RBcDNA or vector alone] were treated with HA (50µg/ml) [or

pre-treated with rat anti-CD44 followed by HA treatment (50 μ g/ml); or treated with no HA. Cell membranes of these cells were solubilized in buffer A [1.0% Nonidet P-40 (NP-40), 1.0% Na₃VO₄, 1.0% phenylmethylsulfonyl fluoride, Okadaic acid (25 ng/ml) in 0.1M phosphate-saline (pH 7.4) and CompleteTM protease inhibitors (Roche)] at 4°C for 1h followed by immunoprecipitation with anti-PI3 kinase (e.g. anti-p110 α or anti-p110 β or anti-p110 γ or anti-p110 δ)-conjugated beads. To measure PI3 kinase activity, anti-p110 α (or anti-p110 β , or anti-p110 γ or anti-p110 δ)-conjugated beads were incubated in 50 μ l buffer A containing 20 μ g of sonicated PIP₂ (Sigma Co.) and 10 μ Ci [γ -³²P]-ATP for 30 min at 32°C. Subsequently, the reaction was terminated by an addition of acidified chloroform:methanol (1:1,v/v). Extracted lipids were then spotted onto a silica Thin Layer Chromatography (TLC) G60 plate (20 x 20 cm; Whatman-precoated with potassium oxalate) and developed using a solvent system containing chloroform/acetone/methanol/acetic acid/water (80:30:26:24:14). The TLC plates were dried and radioactively labeled PIP₃ spots were visualized by autoradiography.

Measurement of M-CSF Production: MDA-MB-231 cells [untransfected (treated with or without LY294002) or transfected with ROK-RBcDNA or vector alone] were washed three times with serum free DMEM and incubated in 3 ml of SF-DMEM containing various reagents [e.g. HA (50 μ g/ml) or anti-CD44 antibody plus HA (50 μ g/ml) or without HA treatment] for 24 hr at 37°C in a 5% CO₂ humidified chamber. Subsequently, M-CSF concentrations in the conditioned medium and cells were determined using the Quantikine M-CSF immunoassay (R & D Systems). Statistical analysis was done using the Student t-test. All data were expressed as the mean \pm SD.

In Vitro Tumor Cell Growth Assays: MDA-MB-231 cells [untransfected (treated with or without LY294002) or transfected with ROK-RBcDNA or vector alone] (5 x 10³ cells/well) were treated with HA (50 μ g/ml) [or pre-treated with rat anti-CD44 followed by HA treatment (50 μ g/ml); or untreated]. These cells were then plated in 96-well culture plates in 0.2ml of Dulbecco's modified Eagle's medium/F12 medium supplement (GIBCO, Grand Island, NY) containing either 0.5% fetal bovine serum or no serum for 24h at 37°C in 5%CO₂/95% air. In each experiment, a total of 5 plates [10 wells/treatment (e.g. HA treatment or rat anti-CD44 plus HA treatment or no treatment)/plate] were used. Experiments were repeated three times. The *in vitro* growth of these cells were analyzed by measuring increases in cell number using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays (CellTiter 96^R non-radioactive cell proliferation assay according to the procedures provided by Promega, Co.) (2). Subsequently, viable cell-mediated reaction products were recorded by a Molecular Devices (Spectra Max 250) ELISA reader at a wavelength of 450nm.

Tumor Cell Invasion Assays: Twenty-four transwell units were used for monitoring *in vitro* tumor cell invasion as described previously (56,57). Specifically, the 5 μ m porosity polycarbonate filters coated with the reconstituted basement membrane substance Matrigel (Collaborative Research, Lexington, MA) were used for the cell invasion assay (56,57). MDA-MB-231 cells [untransfected (treated with or without LY294002) or transfected with ROK-RBcDNA or vector alone] [1 x 10⁴ cells/well in phosphate buffered saline (PBS), pH 7.2] were placed in the upper chamber of the transwell unit in the presence or absence of HA. The growth medium containing high glucose DMEM supplemented by 10% fetal bovine serum were placed in the lower chamber of the transwell

unit. After 18h incubation at 37°C in a humidified 95% air/5% CO₂ atmosphere, cells on the upper side of the filter were removed by wiping with a cotton swap. Cell invasion processes were determined by measuring the cells that migrate to the lower side of the polycarbonate filters by standard cell number counting methods as described previously (15,56,57). Each assay was set up in triplicate and repeated at least 5 times. All data was analyzed statistically by Student's t test and statistical significance was set at $p < 0.01$.

RESULTS

Interaction of CD44v3 and p115RhoGEF in Breast Tumor Cells (MDA-MB-231 Cells):

The level of CD44v3 isoform expression increases as the histologic grade of the breast tumor progresses. In fact, there is a direct correlation between CD44v3 isoform expression and increased histologic grade of the malignancy (22). Using anti-CD44v3 antibody and immunoblot analysis, we have confirmed the presence of the 85kDa CD44v3 protein in MDA-MB-231 cells (Fig. 1, lane 1). A number of studies have shown that CD44v3 and RhoGTPases (in particular, RhoA) are structurally and functionally coupled in breast tumor cells (22). Although several RhoA-specific guanine nucleotide exchange factors (RhoGEFs) have been shown to be involved in RhoA signaling (33,34,58), identification of the specific RhoGEF protein that plays a direct role in regulating CD44v3-specific metastatic behaviors in breast tumor cells is addressed in this study.

Immunoblotting with anti-p115RhoGEF antibody indicates that a single polypeptide (M. W. ~115 kDa) is expressed in MDA-MB-231 cells (Fig. 1, lane 2). In addition, we have carried out anti-p115RhoGEF-mediated or anti-CD44v3-mediated immunoprecipitation followed by anti-CD44v3 immunoblot (Fig. 1, lane 3) or anti-p115RhoGEF immunoblot (Fig. 1, lane 4), respectively. Our results clearly indicate that the CD44v3 is present in anti-p115RhoGEF-immunoprecipitated materials (Fig. 1, lane 3). Conversely, the p115RhoGEF band is also detected in the anti-CD44v3-immunoprecipitated materials (Fig. 1, lane 4). The results with control samples confirm the specificity of these immunological techniques. For example, very little p115RhoGEF is detected in anti-CD44v3-mediated immunoprecipitated materials blotted by an anti-p115RhoGEF-free serum (anti-p115RhoGEF antibody pre-absorbed by an excess amount of p115RhoGEF) (Fig. 1, lane 5). Similarly, no CD44v3 is observed in anti-p115RhoGEF-mediated immunoprecipitated materials blotted by an anti-CD44v3-free serum (anti-CD44v3 antibody pre-absorbed by an excess amount of CD44v3) (Fig. 1, lane 6). These findings indicate that CD44v3 and p115RhoGEF are physically associated in breast tumor cells.

Analyses of p115RhoGEF-Catalyzed RhoA Activation in MDA-MB-231 Cells:

It has been reported that p115RhoGEF functions as an exchange factor for Rho-like GTPases such as RhoA in G-protein-coupled receptor activation (22,33,34). To confirm that the p115RhoGEF-like molecule in this study functions as a GDP/GTP exchange factor [or a GDP-dissociation stimulator (GDS) protein] for RhoGTPases in breast tumor cells, we first isolated p115RhoGEF from MDA-MB-231 cells using anti-p115RhoGEF-conjugated Sepharose beads. As indicated in Fig. 2, the purified p115RhoGEF activates GDP/GTP exchange on GST-tagged RhoA

(Fig. 2). The initial onset of the exchange reaction on GST-tagged RhoA occurs within 1/2-2 min after the addition of p115RhoGEF; and the reaction reaches its maximum approximately 6 min after p115RhoGEF addition (Fig. 2). Most importantly, we have observed that the addition of HA to CD44v3-containing MDA-MB-231 cells stimulates both the rate of p115RhoGEF-mediated GDP/GTP exchange reaction and the maximal amount of bound [35 S]GTP γ S to GST-RhoA (at least a 1.75-fold increase) (Fig. 2a) as compared with the p115RhoGEF isolated from MDA-MB-231 cells without HA treatment (Fig. 2b). In control samples, a low level of [35 S]GTP γ S-bound material was detected in GST alone under same GDP/GTP exchange reaction using p115RhoGEF isolated from MDA-MB-231 cells in the presence (Fig. 2c) or absence (Fig. 2d) of HA treatment. Therefore, we conclude that p115RhoGEF in MDA-MB-231 cells functions as a GDP/GTP exchange factor for Rho-like GTPases such as RhoAGTPase.

Identification of Gab-1 as a New Cellular Substrate for RhoA-activated Rho-Kinase (ROK):

Several studies indicate that Rho-Kinase (ROK-also called Rho-associated kinase-a serine-threonine kinase) interacts with RhoA in a GTP-dependent manner during HA/CD44-mediated cell activation (22,45). Using a ROK-specific antibody, we have confirmed the presence of ROK (~160kDa polypeptide) in MDA-MB-231 cells (Fig. 3, lane 2). No ROK-containing material is observed in control samples when pre-immune rabbit serum is used in these experiments (Fig. 3, lane 1). This RhoA-activated ROK has been shown to induce phosphorylation of a number of cytoskeletal proteins and signaling molecules (22,41-45). A recent study indicates that overexpression of an active form of RhoA (RhoA^{v14}) promotes ROK-mediated serine phosphorylation of insulin-receptor substrate-1 (IRS-1) during insulin signaling (59). In this study we have found that a member of the IRS family, Gab-1, is expressed in MDA-MB-231 cells (Fig. 4A-c). Furthermore, our data indicate that ROK isolated from MDA-MB-231 cells is capable of phosphorylating the Gab-1 at both serine (Fig. 4A-a, lane 3) and threonine residues (Fig. 4A-b, lane 3) (as detected by anti-phospho-serine and anti-phospho-threonine-mediated immunoblot, respectively followed by reblotting with anti-Gab-1 antibody (Fig. 4c, lane 3) in the presence of activated GTP γ S●RhoA (Figs. 4A-a, 4A-b and 4A-c, lane 3). The level of serine/threonine phosphorylation of Gab-1 appears to be relatively low if ROK was incubated with unactivated RhoA (RhoA without GTP γ S bound) (Figs. 4A-a, 4A-b and 4A-c, lane 2). In the absence of ROK, no serine/threonine phosphorylation of Gab-1 is detected (Figs. 4A-a, 4A-b and 4A-c, lane 1). These results indicate that Gab-1 serves as one of the cellular substrates for the RhoA-dependent kinases such as ROK *in vitro*.

Furthermore, we have examined the effect of ROK-mediated Gab-1 phosphorylation on CD44 binding. Specifically, the highly phosphorylated form of Gab-1 [by ROK plus activated RhoA (RhoA with GTP γ S bound, GTP γ S●RhoA)] (as shown in Fig. 4A, lane 3) or the minimally phosphorylated form of Gab-1 [using ROK plus unactivated RhoA (e.g. RhoA without GTP γ S bound) (as shown in Fig. 4A, lane 2) was incubated with various concentrations of 125 I-labeled CD44 cytoplasmic domain (FLAG-CD44cyt) under equilibrium binding conditions. Our results indicate that CD44 binds to either highly phosphorylated Gab-1 or minimally phosphorylated Gab-1 at a single site. Importantly, the highly phosphorylated Gab-1 displays at least 3-fold higher CD44 binding affinity (with an apparent K_d ~0.18nM) (Fig. 4B-a) than the minimally phosphorylated Gab-1 (with an apparent K_d ~0.58nM) (Fig. 4B-b). These results clearly support the notion that Gab-1

phosphorylation by RhoA-activated ROK enhances its binding interaction with CD44 which may be required for Gab-1-mediated recruitment of signaling components into CD44-associated membrane during HA activation. Other approaches, such as mutational analyses of the appropriate serine/threonine residues on Gab-1 (activated by RhoA-activated ROK) required for CD44 binding, are under investigation in our laboratory.

Previous studies determined that HA-CD44 interaction promotes ROK activation in breast tumor cells (22). Several cellular proteins including the cytoplasmic domain of CD44 and IP₃ receptors have been identified as ROK-specific cellular substrates during HA-CD44 signaling (45). Overexpression of a dominant-negative form of ROK [by transfecting cells with ROK's Rho-Binding domain (ROK-RB)cDNA] is capable of inhibiting endogenous ROK activity leading to downregulation of HA/CD44 signaling and tumor-specific phenotypes (22). In order to demonstrate ROK-mediated phosphorylation of Gab-1 *in vivo*, a dominant-negative form of ROK [ROK's Rho-Binding (ROK-RB)]cDNA was cloned into a green fluorescent protein (GFP)-tagged expression vector (pEFGPC1 vector) followed by a transient transfection of GFP-RB cDNA into MDA-MB-231 cells. Anti-GFP-mediated immunoblot analysis indicates that the ROK's RB fragment is expressed in MDA-MB-231 cells transfected with ROK's RBcDNA (Fig. 5A-a, lane 4-6), but not in those cells transfected with vector alone (Fig. 5A-a, lane 1-3). Using anti-ROK-mediated immunoblot, we have detected no significant difference in endogenous ROK expression between ROK's RBcDNA-transfected (Fig. 5A-a, lane 4-6) and vector-transfected cells (Fig. 5A-a, lane 1-3) treated with HA or no HA (or pretreated with anti-CD44 followed by HA treatment). The question of whether Gab-1 phosphorylation and Gab-1-mediated signaling events are affected by HA/CD44-activated ROK and/or the dominant-negative form of ROK (ROK-RB overexpression) in breast tumor cells is the focus of this study.

The results of our analyses indicate that the level of serine and threonine phosphorylation of Gab-1 [as detected by anti-Gab-1-mediated immunoprecipitation followed by immunoblotting with anti-serine (Fig. 5B-a) or anti-threonine (Fig. 5B-b) or anti-Gab-1 (Fig. 5B-c), respectively (Fig. 5B)] is greatly enhanced in vector-transfected cells treated with HA (Fig. 5B-a, lane 2; Fig. 5B-b, lane 2). In contrast, Gab-1 serine and threonine phosphorylation is relatively low in vector-transfected cells without any HA treatment (Fig. 5B-a, lane 1; Fig. 5B-b, lane 1) or those vector-transfected cells pretreated with anti-CD44 followed by HA treatment (Fig. 5B-a, lane 3; Fig. 5B-b, lane 3). These observations strongly support the conclusion that HA-mediated Gab-1 phosphorylation is CD44-dependent. It is also noted that transfection of MDA-MB-231 cells with ROK's RBcDNA does not alter the basal level of Gab-1 serine and threonine phosphorylation in transfectants without HA treatment (Fig. 5B-a, lane 4; Fig. 5B-b, lane 4) or transfectants pre-treated with anti-CD44 followed by HA treatment (Fig. 5B-a, lane 6; Fig. 5B-b, lane 6). However, overexpression of ROK's RB appears to greatly reduce the ability of Gab-1 to respond to HA-mediated serine (Fig. 5B-a, lane 5) and threonine phosphorylation (Fig. 5B-b, lane 5). These findings suggest that ROK is closely involved in the regulation of HA/CD44-mediated Gab-1 phosphorylation *in vivo*.

HA-CD44-Mediated Activation of Gab-1-Linked PI3 Kinase Signaling:

Gab-1 is known to play an important role as a linker molecule that interacts with several signaling molecules including PI3 kinase (46-48). Gab-1 also acts to potentiate and diversify signals downstream from receptors by virtue of its ability to assemble multiprotein complexes (47,48). In addition, phosphorylation of Gab-1 has been shown to play an important role in membrane localization and activation of PI3 kinase (46-48). We next examined the potential impact of ROK-mediated Gab-1 phosphorylation on the regulation of PI3 kinase in MDA-MB-231 cells transfected with ROK's RBcDNA or vector alone. Using anti-Gab1-mediated immunoprecipitation of MDA-MB-231 cell lysates (isolated from ROK's RBcDNA or vector-transfected cells) followed by immunoblotting with various PI3 kinase antibodies [e.g. anti-PI3 kinase (α , β , γ and δ form of p110 catalytic subunit antibody)], we have found that all four PI3 kinases [such as p110 α (Fig. 6a, lane 1-4), p110 β (Fig. 6b, lane 1-4), p110 γ (Fig. 6c, lane 1-4) and p110 δ form (Fig. 6d, lane 1-4)] are equally co-precipitated with Gab-1 in these transfectants either with (Fig. 6a,b,c,d, lane 2 & 4) or without any HA treatment (Fig. 6a,b,c,d, lane 1 & 3). Thus, we believe that both the highly phosphorylated (Fig. 5B-a, lane 2; Fig. 5B-b, lane 2) and minimally phosphorylated forms of Gab-1 (Fig. 5B-a, lane 1,3,4,5 & 6; Fig. 5B-b, lane 1,3,4,5 & 6) are capable of binding to PI3 kinases (e.g. α , β , γ and δ form of p110 catalytic subunit of PI3 kinase) *in vivo* (Fig. 6).

Many reports have shown that certain adaptor molecules such as Gab-1 are involved in bringing p110 to the plasma membrane during cellular signaling (46-51). Gab-1 contains several functional domains including the pleckstrin homology (PH) domain (Fig. 7A-a) which may mediate association with the submembrane region of the cell via protein-protein or protein-lipid interactions (35,36). To determine whether the PH domain of Gab-1 is involved in a direct interaction with CD44, we have prepared a purified recombinant Gab-1-PH fragment (conjugated to Sepharose beads) and FLAG-tagged cytoplasmic domain of CD44 (FLAG-CD44cyt) fusion protein for *in vitro* binding analysis. Our results show that FLAG-CD44cyt is tightly associated with Gab-1-PH-His fragment-conjugated beads (Fig. 7A-b, lane 2) but not with His-conjugated beads (Fig. 7A-b, lane 1). These observations establish the fact that the PH domain of Gab-1 may be responsible for the recognition of CD44 *in vitro*.

In order to further analyze the interaction between the PH domain of Gab-1 and CD44v3 *in vivo*, we have used the Gab-1-PH fragment construct that encodes the sequence between aa5-aa117 of Gab-1's PH domain (Fig. 7A-a and 7A-b). This construct was then cloned into a His-tagged expression vector followed by a transient transfection of His-tagged PHcDNA (or vector alone) into MDA-MB-231 cells. When these transfectants were immunoprecipitated by anti-CD44v3 antibody followed by immunoblotting with anti-CD44v3 and anti-His, respectively, we have determined that the His-tagged PH fragment of Gab-1 (Fig. 7B-f, lane 3 and 4) is co-precipitated with CD44v3 (Fig. 7B-g, lane 3 and 4) from cells transfected with His-Gab-1-PHcDNA (in the presence or absence of HA) (Fig. 7B, lane 3 and 4). No detectable cellular protein (Fig. 7B-f, lane 1 and 2) is found in anti-CD44v3-mediated immunoprecipitated/immunoblotted materials (Fig. 7B-g, lane 1 and 2) isolated from vector-transfected cells treated with HA or no HA (Fig 7B, lane 1 and 2). These results indicate that the PH domain of Gab-1 is closely complexed with CD44v3 *in vivo*.

Moreover, we have demonstrated that HA is capable of promoting the recruitment of endogenous Gab-1 (Fig. 7B-e, lane 1 and 2) together with various PI3 kinases (e.g. p110 α , p110 β , p110 γ and p110 δ) (Fig. 7B-a,b,c and d, lane 1 and 2) into a complex with CD44v3 (Fig. 7B-g, lane 1 and 2) in vector-transfected cells (Fig. 7B, lane 1 and 2). In contrast, transfection of MDA-MB-231 cells with Gab-1's PHcDNA not only causes significant reduction in endogenous Gab-1 (Fig. 7B-e, lane 3 and 4) association with CD44v3 (Fig. 7B-g, lane 3 and 4) but also exhibits a marked inhibition in HA-mediated recruitment of Gab-1-linked PI3 kinases (Fig. 7B-a,b,c and d, lane 3 and 4) to CD44v3 (Fig. 7B-g, lane 3 and 4). These findings suggest that the Gab-1 fragment containing the PH domain acts as a potent competitive inhibitor for endogenous intact Gab-1 binding to CD44v3 *in vivo*; and it also functions as a strong dominant-negative mutant for blocking CD44-Gab-1-mediated PI3 kinase membrane localization.

We have also investigated the effects of ROK-RB overexpression (by transfecting cells with ROK's RBcDNA) on CD44v3 interaction with Gab-1 and PI3 kinase. Using anti-CD44v3 immunoprecipitation of MDA-MB-231 cells followed by immunoblotting with anti-Gab-1, we have confirmed that both CD44v3 and Gab-1 are physically linked as a complex in vector-transfected cells without HA treatment (Fig. 8A-e and f, lane 1). However, HA treatment of vector-transfected cells appears to promote a significant recruitment of Gab-1 into CD44v3 (Fig. 8A-e and f, lane 2). Moreover, we have used anti-CD44v3-mediated immunoprecipitation followed by immunoblotting with various anti-PI3 kinase antibodies (e.g. anti- α , β , γ and δ form of p110 catalytic subunit of PI3 kinase). Our results show that a relatively low level of some PI3 kinase (e.g. p110 γ and p110 δ form but not p110 α & p110 β) is co-precipitated with CD44v3 in vector-transfected cells without HA treatment (Fig. 8A-a,b,c,d,e & f, lane 1). However, HA treatment of vector-transfected cells appears to enhance the recruitment of PI3 kinase (including p110 α , p110 β , p110 γ and p110 δ) into CD44v3 (Fig. 8A-a,b,c,d,e & f, lane 2). We have also determined that the basal level of Gab-1/PI3 kinase (p110 γ and p110 δ) association with CD44v3 is greatly reduced in ROK's RBcDNA-transfected cells (without HA treatment) (Fig. 8A-a,b,c,d,e & f, lane 3). Most noticeably, overexpression of ROK-RB in MDA-MB-231 transfectants abolishes the ability of HA to recruit Gab-1-linked PI3 kinase (Fig. 8A-a,b,c,d,e & f, lane 4). It is therefore possible that HA/CD44-activated ROK plays an important role in the recruitment of Gab-1-PI3 kinase complexes to certain plasma membrane proteins such as CD44v3 in breast tumor cells.

In addition, we have examined Gab-1-linked PI3 kinase activity in anti-p110 α , β , γ and δ form-specific immunoprecipitates (obtained from membrane fraction of vector-transfected or ROK's RBcDNA-transfected cells treated with HA or without any HA) (Fig. 8B). Our results indicate that activation of PI3 kinase [in particular, p110 α (Fig. 8B-a, lane 1 and 2), p110 β (Fig. 8B-b, lane 1 and 2) and p110 γ (Fig. 8B-c, lane 1 and 2) but not p110 δ (Fig. 8B-d, lane 1 and 2)] occurs as early as 5 min after HA addition to vector-transfected cells, resulting in an increase in the level of PtdIns (3,4,5)-P₃ formation. However, HA-induced increase in PtdIns (3,4,5)-P₃ by Gab-1-associated PI3 kinases [in particular, p110 α (Fig. 8B-a, lane 3 and 4) and p110 β (Fig. 8B-b, lane 3 and 4)] appears to be significantly inhibited in ROK's RBcDNA-transfected cells. Thus, it is likely that activation of both p110 α and p110 β are HA/CD44 and ROK-dependent. Although HA is capable of up-regulating p110 γ activity (Fig. 8B-c, lane 1 and 2), the failure of blocking HA-activated p110 γ activity in ROK's RBcDNA-transfected cells (Fig. 8B-c, lane 3 and 4) supports the possibility that p110 γ

stimulated by HA is ROK-independent. Finally, we have noted that HA fails to activate p110 δ activity in both vector-transfected (Fig. 8B-d, lane 1 and 2) and ROK's RBcDNA-transfected cells (Fig. 8B-d, lane 3 and 4), suggesting that p110 δ activity is not regulated by HA/CD44-mediated ROK signaling pathway in breast tumor cells.

Effects of RhoA-Activated ROK and Gab-1-Linked PI3 Kinase on AKT Activation, Cytokine (M-CSF) Production and Breast Tumor Cell Growth and Invasion:

Activation of AKT by growth factors is known to be mediated by PI 3-kinase (60-62). Most importantly, AKT acts as an important mediator of many cell survival-signaling pathways and is often overexpressed/amplified in tumor progression (60-62). In this study we have investigated the possible role of ROK and PI3 kinase in regulating AKT-1 activation in breast tumor cells during HA-CD44 signaling. Specifically, we have determined that AKT-1 kinase is significantly stimulated by HA treatment in vector-transfected cells (detected by anti-phospho-AKT-1 (Fig. 9a, lane 1) followed by reblotting with anti-AKT-1 antibody) (Fig. 9b, lane 2) as compared to the level of AKT-1 phosphorylation in vector-transfected cells without any HA treatment (Fig. 9a and b, lane 1). Moreover, treatment of vector-transfected cells with a PI3 kinase inhibitor (LY294002) results in down-regulation of AKT-1 activation during the response to HA treatment (Fig. 9a and b, lane 3 and 4). Furthermore, HA-activated AKT-1 phosphorylation is also greatly reduced in ROK's RBcDNA-transfected cells (Fig. 9a and b, lane 5 and 6) comparing with AKT-1 phosphorylation in vector-transfected cells treated with HA treatment (Fig. 9a and b, lane 1 and lane 2). These observations strongly support the conclusion that both PI3 kinase signaling and ROK activation are important upstream activators for AKT signaling required for HA/CD44-mediated tumor cell survival.

As part of our effort to identify new effector functions influenced by HA/CD44-mediated ROK and PI3 kinase signaling in breast tumor cells, we decided to examine the production of M-CSF which is a homodimeric cytokine in the colony-stimulating factor family (63). In breast cancers, M-CSF serum levels correlate with prognosis, activity and invasiveness of the disease (64). Here, we have found that HA promotes M-CSF production in untransfected MDA-MB-231 cells (Table 1A). It is also noted that the amount of M-CSF production detected in the media of untransfected MDA-MB-231 cells pre-treated with rat anti-CD44 (but not normal rat IgG) followed by HA treatment was significantly reduced (Table 1A). These results support the notion that the stimulation of M-CSF production by HA is CD44-dependent. Furthermore, treatment of untransfected cells with a PI3 kinase inhibitor, LY294002 blocks HA-mediated M-CSF production (Table 1B). Moreover, our data show that HA/CD44-activated and PI3 kinase-regulated M-CSF production are significantly inhibited in MDA-MD-231 cells transfected with ROK's RB cDNA as compared to that detected in vector-transfected cells (Table 1C). Together, these findings suggest that both RhoA-ROK and PI3 kinase activation are closely involved in HA/CD44-mediated M-CSF production in breast tumor cells.

Furthermore, using *in vitro* growth and invasion assays, we have found that CD44v3-containing MDA-MB-231 cells undergo active cell growth (Table 2A) and invasion (Table 2B). HA activates both breast tumor cell growth (Table 2A) and invasion (Table 2B). In contrast, MDA-MB-231 cells pretreated with rat anti-CD44 (but not normal rat IgG) followed by HA addition greatly reduces their ability to undergo growth and invasion (data not shown). These observations suggest

that HA-mediated tumor cell growth and invasion is also CD44-dependent. Treatment of MDA-MB-231 cells with various agents such as the PI3 inhibitor (LY294002), causes a significant inhibition of HA-mediated tumor cell growth (Table 2A) and invasion (Table 2B). Importantly, transfection of MDA-MB-231 cells with the dominant-negative form of ROK (ROK-RB)cDNA (but not vector alone) also effectively blocks HA/CD44-mediated breast tumor cell growth (Table 2A) and invasion (Table 2B). Together, these findings support the notion that HA-mediated CD44 signaling is functionally linked to RhoA-ROK and Gab-1-linked PI3 kinase activation required for metastatic breast tumor progression.

KEY RESEARCH ACCOMPLISHMENTS:

- We have found that Rho-Kinase (ROK) is one of the known RhoA-activated enzymes and is expressed in metastatic breast tumor cells (e.g. MDA-MB-231 cell lines).
- Our data indicate that the interaction between the metastasis-specific molecule, CD44 [a hyaluronan (HA) receptor] and RhoA-specific guanine nucleotide exchange factors (RhoGEF) promotes ROK activation in metastatic breast tumor cells (MDA-MB-231 cell line).
- We have identified that Gab-1 (Grb-2-associated binder-1) as a new cellular substrate for ROK in breast tumor cells. Specifically, ROK is capable of phosphorylating Gab-1 which serves as a linker protein for recruiting signaling molecules into the plasma membrane.
- We have demonstrated that ROK-phosphorylated Gab-1 promotes PI3 kinase recruitment to CD44. Subsequently, PI3 kinase is activated (in particular, α , β , γ forms but not the δ form of the p110 catalytic subunit), AKT signaling occurs, the cytokine [macrophage-colony stimulating factor (M-CSF)] is produced and tumor cell-specific phenotypes (e.g. tumor cell growth, survival and invasion) are upregulated.
- Our results also indicate that overexpression of a dominant-negative form of ROK [by transfection of MBA-MD-231 cells with ROK's Rho-binding (RB) domain cDNA] not only inhibits HA/CD44-mediated RhoA-ROK activation and Gab-1 phosphorylation, but also downregulates oncogenic signaling events (e.g. Gab-1/PI3 kinase-CD44v3 association, PI3 kinase-mediated AKT activation and M-CSF production) and tumor cell behaviors (e.g. cell growth, survival and invasion).
- These observations strongly suggest that CD44 interaction with p115RhoGEF and ROK plays a pivotal role in promoting Gab-1 phosphorylation leading to Gab-1-PI3 kinase membrane localization, AKT signaling and cytokine (M-CSF) production during HA-mediated breast cancer progression.

REPORTABLE OUTCOMES:

- Manuscripts and abstracts: see section #13 (final report section).
- Funding applied for based on work supported by this award:

Funded Active Grants:

NCI Grant (2000-2005) "CD44/Variant-Cytoskeleton In Breast Cancer Progression".

NCI Grant (1999-2008) "CD44-p185^{HER2} Interaction In Ovarian Cancer Progression".

NCI Grant (2001-2003) "CD44 Interaction With Cytokine Receptors in Breast Cancer Bone Metastasis".

VA Merit Award Grant (2003-2008) "CD44-EGF Receptor Signaling in Head & Neck cancer Progression".

US Army Breast Cancer Grant (DOD) (1999-2002) "A Novel Signaling Perturbation and Ribozyme Gene Therapy Procedures to Block Rho-Kinase (ROK) Activation and Breast Tumor Metastasis".

CONCLUSIONS:

CD44 belongs to a family of transmembrane glycoproteins [e.g. CD44s (standard form), CD44E (epithelial form) and CD44v (variant isoforms)] which are often overexpressed in a variety of human solid neoplasms including breast cancers (1-6). In particular, the CD44v3-containing isoforms are expressed preferentially in highly malignant breast carcinoma tissue samples (1-6). In breast tumor cells, CD44v3 has a heparin sulfate addition site in the membrane-proximal extracellular domain of the molecule that confers the ability to bind vascular epithelial growth factor (VEGF), but not FGF-2 (13). The attachment of VEGF to the heparin sulfate sites on CD44v3 may be responsible for the onset of breast tumor-associated growth, invasion and angiogenesis. The level of CD44v3 isoform expression often increases as the histological grade of each of the breast tumors progresses. In fact, there is a direct correlation between CD44v3 isoform expression and increased histologic grade of the malignancy (1-6). These lines of evidence suggest that expression of certain CD44v3 isoform(s) may be an accurate predictor of eventual survival (including nodal status, tumor size, and grade) during breast cancer progression (4). It is now well established that the extracellular matrix component, hyaluronan (HA), is one of the primary ligands recognized by surface CD44 molecules including CD44v3 (21-24). Both HA and CD44v3 appear to be overexpressed at sites of tumor attachment, and are known to be involved in tumor cell-specific properties (e.g. tumor cell growth, survival, migration and invasion) (21-24). In this study we have found that CD44v3 is expressed as a 85kDa polypeptide in breast tumor cells [MDA-MB-231 cells (Fig. 1)]. The question of which oncogenic pathways are directly involved in regulating HA-activated and CD44v3-specific breast tumor cell behaviors is addressed in this study.

The invasive phenotype of breast tumor cells, characterized by HA-CD44v3-mediated "invadopodia" formation (57), MMP-9 activation (57), tumor cell migration and invasion (21-24; 56,57) has been linked to cytoskeletal function, a process in which the small GTP-binding proteins such as RhoA are known to play important roles (22). The activities of members of the RhoGTPases

(including RhoA) are often regulated by guanine nucleotide exchange factors (GEFs) that contain a dbl homology (DH) domain (33,34). In recent years, a RhoA-specific GEF (p115RhoA) molecule containing several functional domains [an amino-terminal RGS domain, a Dbl homology (DH) domain and a pleckstrin homology (PH) domain] have been identified and characterized (33,34). In particular, the DH domain of p115RhoGEF by itself exhibits GDP/GTP exchange activity for RhoA, and plays an important role in RhoGTPase signaling (33,34). Unique to p115RhoGEF, compared to other GEFs for small GTPases, is the presence of a RGS domain. A number of laboratories have reported that the N-terminal RGS domain interacts with the α subunit (α 12/13) of the G-protein (33,34). In fact, the RGS sequence has been shown to play an important role in regulating p115RhoGEF-G α 12/13 interactions during G-protein-coupled receptor signaling (33,34). Our laboratory has demonstrated that p115RhoGEF is expressed in MDA-MB-231 cells (Fig. 1). In addition, we have presented new evidence for a close, physical interaction between p115RhoGEF and the transmembrane glycoproteins, CD44v3 isoform (Fig. 1). Most importantly, our results show that the binding of HA to CD44v3 promotes p115RhoGEF-catalyzed RhoA activation (Fig. 2). Therefore, it is likely that CD44v3 serves as an activator of p115RhoGEF function. Clearly, these two proteins, CD44v3 and p115RhoGEF, are not only structurally linked, but also functionally coupled. It is also noted that two other RGS-containing RhoGEF's (e.g. PDZ-RhoGEF and/or LARG) have been shown to activate RhoA signaling (34,58). The relationship between PDZ-RhoGEF and/or LARG in HA/CD44-mediated cellular events remains to be elucidated.

Rho-kinase (ROK) stimulated by activated RhoA (GTP-bound form of RhoA) appears to play a pivotal role in promoting cytoskeletal function and a variety of other cellular activities (22,40-45). In a previous study we demonstrated that ROK phosphorylates the cytoplasmic domain of CD44v_{3,8-10} isoform and up-regulates the interaction between CD44v_{3,8-10} isoform and the cytoskeletal protein, ankyrin, during HA/CD44-regulated tumor cell migration (22). More recently, we have shown that the pleckstrin homology (PH) domain of ROK is the primary binding site for CD44 (45). Most importantly, our results indicate that CD44 interaction with ROK plays a pivotal role in the phosphorylation of inositol triphosphate receptor (IP₃ receptor) leading to an increase of IP₃ binding followed by a rapid stimulation of Ca²⁺ mobilization during HA-mediated cell migration (45). Therefore, we believe that ROK plays an important role in HA/CD44-mediated functions (45). Structurally, ROK is composed of catalytic (CAT), coiled-coil, Rho-binding (RB) and pleckstrin-homology (PH) domains (37-40). A number of substrates, including myosin-binding subunit (MBS) of myosin phosphatase (41), calponin (42), adducin (43), LIM kinase (44), IP₃ receptors (45) and CD44 (22), have been shown to be phosphorylated by ROK. In MDA-MB-231 cells, we have identified a 160 kDa protein as a ROK molecule (Fig. 3). In an effort to identify possible new cellular substrates for ROK, we have focused on the multiple docking protein, Gab-1, that is often tyrosine phosphorylated by receptor tyrosine kinases such as hepatocyte growth factor receptor, c-Met (65) and epidermal growth factor receptors (66). In this study we have demonstrated that ROK (by binding to activated RhoA) is capable of inducing marked serine and threonine phosphorylation of Gab-1 (isolated from MDA-MB-231 cells) *in vitro* (Fig. 4). The ability of ROK to phosphorylate Gab-1 in the presence of unactivated RhoA appears to be greatly reduced (Fig. 4). These results clearly indicate that ROK acts as one of the downstream effectors of RhoA signaling and utilizes Gab-1 as one of its cellular targets *in vitro*. Overexpression of the Rho-binding (RB) domain of ROK has been found to function as a dominant-negative form of ROK which inhibits ROK activity, ROK-

mediated protein phosphorylation and HA/CD44-mediated breast tumor progression (22). This information has prompted us to examine whether ROK plays a role in regulating Gab-1 phosphorylation and Gab-1-mediated cell signaling in HA/CD44-mediated breast tumor cell activation *in vivo*. Transfection of MDA-MB-231 cells with ROK's RB domaincDNA (Fig. 5), which effectively competes for endogenous activated RhoA binding to ROK and inhibits the ability of ROK to phosphorylate Gab-1 during HA-CD44 signaling (Fig. 5), strongly suggests that ROK's RB is a potent inhibitor of ROK-mediated Gab-1 phosphorylation *in vivo*.

A number of studies have shown that tyrosine phosphorylated Gab-1 is involved in PI3 kinase binding, membrane translocation and activation (47,48,67,68). The question of whether HA/CD44-activated RhoA-ROK serine/threonine phosphorylation of Gab-1 plays a role in regulating PI3 kinase signaling is addressed in this study. Previous reports have indicated that coexpression of multiple PI3 kinase isoforms (e.g. α , β , γ and δ form of p110) occurs in many different cells (46,49-51). The p110 α , p110 β and p110 γ forms are expressed in all tissue, whereas p110 δ is preferentially expressed in leukocytes (46,49-51). The α , β and δ forms of the p110 catalytic subunit belongs to the class IA PI3 kinase known to transmit signals from tyrosine kinase-coupled receptors (66,67). These class IA PI3 kinases (e.g. p110 α , p110 β and p110 δ) are often associated with certain regulatory units (e.g. p85, p55 or p50) that bind to specific phosphorylated tyrosine residues in receptor proteins or other adaptor molecules such as Gab-1 (66,67). The class IB PI3 kinase (the γ form of p110 catalytic subunit) can be activated by G-protein-coupled receptors (GPCRs) (46,49-51,67). This p110 γ form interacts with a unique regulatory protein, p101 (but not p85, p55 or p50) (69). Here, we have found that multiple isoforms of PI3 kinase (as detected by immunoblot with specific anti- α , β , γ and δ forms of the p110 catalytic subunit) are also coexpressed in MDA-MB-231 cells (Fig. 6). Apparently, the status of Gab-1 serine/threonine phosphorylation (either highly phosphorylated or unphosphorylated form) does not affect Gab-1 association with any particular isoform of PI3 kinase (e.g. p110 α , p110 β , p110 γ and p110 δ). These results suggest that the physical association between Gab-1 and PI3 kinase is always present and serine/threonine phosphorylation of Gab-1 plays a small role (if any) in influencing Gab-1-PI3 kinase complex formation (Fig. 6). The question of whether Gab-1 (phosphorylated or unphosphorylated form) directly binds to p110 subunits or selectively interacts with various regulatory domains (e.g. p85, p55 or p50 or p101) in HA/CD44-mediated RhoA-ROK signaling awaits further analyses.

In addition, using two recombinant proteins (the PH domain of Gab-1 and the cytoplasmic domain of CD44), we have demonstrated that Gab-1's PH is responsible for the recognition of CD44 *in vitro* (Fig. 7A). We have also provided new evidence that phosphorylation of intact Gab-1 by RhoA-activated ROK enhances its binding to CD44 (Fig. 4B). Furthermore, we have found that transfection of MDA-MB-231 cells with Gab-1-PHcDNA (Fig. 7B) effectively blocks endogenous Gab-1 association with CD44v3 and the subsequent recruitment of signaling molecules (e.g. PI3 kinase) to CD44v3-containing membrane (Fig. 7B). These findings further support our conclusion that Gab-1-PH acts as a potent competitive inhibitor which is capable of interfering with endogenous Gab-1-CD44v3 interaction *in vivo*. These results are consistent with our previous study showing a sequence adjacent to the N-terminal region of PH domain (the PHn-CC-EX domain) of Tiam1 [a Rac1-specific guanine nucleotide exchange factor (GEF)] that is involved in the direct binding to CD44v3 isoform during HA stimulated Rac1 signaling and cytoskeleton-mediated tumor cell

migration (24). Our recent study also shows that the PH domain of ROK binds to CD44v10 in endothelial cells and this ROK-PH interaction with CD44v10 regulates HA-mediated Ca^{2+} signaling and endothelial cell migration (45). It is therefore, apparent that the close interaction between CD44 isoforms and certain PH domain-containing molecules (e.g. Gab-1, Tiam1 and ROK) plays an important role in HA signaling. Moreover, we have observed that the Gab-1 (highly serine/threonine phosphorylated form of Gab-1)-PI3 kinase complex becomes preferentially recruited into CD44v3-containing membranes in vector-transfected cells treated with HA (Fig. 8A). In contrast, in other situations, the Gab-1 (with a minimal level of serine/threonine phosphorylation)-PI3 kinase complex fails to become membrane localized with CD44v3 in vector-transfected cells with no HA treatment (Fig. 8A) or in ROK's RBcDNA-transfected cells with or without HA treatment (Fig. 8A). Thus, our results provide an evidence for HA/CD44-activated RhoA-ROK to play a role in the membrane translocation of phosphorylated Gab-1-PI3 kinase complexes into CD44v3. The recruitment of phosphorylated Gab-1-PI3 kinase into CD44v3-associated membranes may bring the p110 catalytic subunits into close proximity to the inositol lipid substrates and thereby facilitate the onset of enzymatic activity.

Previously, PI3 kinase has been shown to be activated by guanine-nucleotide-binding proteins (70,71). PI3 kinase can also be activated via its binding to the active conformation of Ras during the membrane localization of p110-p85 complex (72,73). Our analyses indicate that ROK-mediated Gab-1 phosphorylation and Gab-1-PI3 kinase membrane localization are accompanied by a robust stimulation of PI3 kinase activity (in particular, α , β and γ forms, but not δ form of p110 catalytic subunit) in vector-transfected cells treated with HA (Fig. 8B). Under physiological conditions, hyaluronan often exists as a high molecular weight polymer ($>10^6$ Daltons). However, generation of hyaluronan fragments may occur during tumor progression. Both HA and certain size HA fragments (3-25 disaccharide units) have been found to either promote up-regulation or down-regulation of CD44-specific biological events (18). Since we have used large size HA ($>10^6$ Daltons) for all experiment described in this study, it is possible that large HA fragments preferentially activate p110 α , p110 β and p110 γ forms, but not p110 δ form in MDA-MB-231 cells (Fig. 8B). The question of whether certain small size HA fragment(s) is(are) capable of promoting p110 δ and/or the same p110 α , p110 β and p110 γ forms, as the native large molecular weight hyaluronan used in this study is currently undergoing investigation in our laboratory.

Recently, Sawyer et al have shown that PI3 kinase (e.g. p110 α , p110 β and p110 δ) is expressed in breast tumor cells (79). In particular, p110 δ (to a lesser extent p110 β , but not p110 α) is involved in epidermal growth factor (EGF)-mediated motility *in vitro* (79). PI3 kinase (such as p110 δ) is known to be activated by tyrosine phosphorylated regulatory subunits (e.g. p85 α , p85 β , and p55 γ) during EGF-mediated EGF receptor tyrosine kinase signaling (46,49-51). Thus, the migratory property associated with p110 δ may be specific for EGF signaling. In this study we have shown that HA promotes recruitment of PI3 kinase p110 δ to CD44v3-containing membrane (Figs. 7 and 8) but does not activate PI3 kinase p110 δ in MDA-MB-231 cells (Fig. 8B). The cytoplasmic domain of CD44 contains no intrinsic tyrosine kinase. Consequently, tyrosine phosphorylation of regulatory subunits (e.g. p85 α , p85 β , and p55 γ) and activation of p110 δ fail to occur during HA-CD44 signaling.

Furthermore, our results indicate that the reduction of Gab-1 threonine/serine phosphorylation and the lack of Gab-1-PI3 kinase membrane localization in ROK's RBcDNA-transfected cells (Fig. 7B) are closely associated with an inhibition of certain PI3 kinase activity (e.g. α and β form but not γ form of p110 catalytic subunit) during HA treatment (Fig. 8). Apparently, the activation of both α and β form of p110 catalytic subunits is ROK-dependent, whereas the stimulation of the γ form of p110 catalytic subunit is ROK-independent (Fig. 8). Moreover, our observations showing that ROK is required for the recruitment of PI3 kinase p110 γ , but ROK is not required for HA-mediated activation of this form (in terms of PIP3 production), are noteworthy. Activation of the catalytic subunit p110 γ is known to be mediated by the regulatory subunit p101. It is possible that membrane localization of PI3 kinase p110 γ is ROK-dependent, whereas p101-mediated activation of PI3 kinase p110 γ is ROK-independent during HA-CD44 signaling. Characterization of p101-mediated activation of p110 γ (in a ROK-independent process) during HA-CD44 signaling awaits further investigation. The fact that HA-mediated PI3 kinase p110 γ recruitment and activation can be readily blocked by anti-CD44 antibody pretreatment of vector and ROK-RBcDNA-transfected cells (data not shown) suggests that CD44 is involved in this HA signaling event. Of course, we can not preclude the possibility that other HA receptors (e.g. RHAMM) are also capable of generating PI3 kinase signaling in either a ROK-dependent or independent manner. The selective activation of α , β and γ forms (but not the δ form of p110 catalytic subunit) may significantly influence the biological outcomes of HA-CD44-mediated oncogenic events in breast tumor progression.

One of the well-established effector molecules for PI3 kinase is AKT which is known as an important mediator of many cell survival-signaling pathways (60-62). Recent evidence has suggested that AKT signaling plays a central role in human malignancy. AKT is a putative oncogene encoding a member of a subfamily of protein-serine/threonine that includes the protein kinase B (PKB) family (60-62). In humans, at least three AKT genes (designated AKT-1, AKT-2 and AKT-3) have been identified (62). It is still unclear how much functional redundancy exists among these three AKTs. In this study we have found that AKT-1 is expressed in MDA-MB-231 cells (Fig. 9) and HA-CD44 interaction promotes AKT-1 activation (Fig. 9). The fact that overexpression of ROK-RB (by transfecting MDA-MB-231 cells with ROK-RBcDNA) impairs HA-mediated AKT-1 activation, and that a PI3 kinase inhibitor (LY294002) also blocks AKT-1 activation (Fig. 9) suggests that both ROK and PI3 kinase are located upstream of AKT-1 and are involved in regulating HA/CD44-mediated AKT signaling required for anti-apoptotic processes and tumor cell survival. Our results are consistent with a previous report showing that HA-CD44 interaction is involved in PI3 kinase/AKT cell survival signaling in human lung carcinoma cells (80). Perturbation of HA-CD44 binding effectively downregulates PI3 kinase/AKT activities and inhibits tumor cell growth (80).

A recent study indicates that breast tumor cells (e.g. MDA-MB-231 cells) are capable of inducing osteoclastogenesis by secreting M-CSF which is a homodimeric cytokine of the colony-stimulating factor family (63). The addition of M-CSF to osteoclast precursors induces the expression of RANK [the receptor for RANKL (the receptor activator of nuclear factor κ B ligand)], which in turn interacts with RANKL and induces differentiation of cells in the macrophage/osteoclast lineage (74-78). In the presence of M-CSF, the RANK/RANKL/OPG (osteoprotegerin) axis mediates osteoclast formation and activity, and thereby bone resorption

(63,74-78). These findings suggest that the stimulation of osteoclastogenesis by M-CSF-producing breast tumor cells is closely involved in osteolytic metastases. In MDA-MB-231 cells we have found that HA promotes M-CSF production in a CD44-dependent manner (Table 1). Inhibition of both ROK and PI3 kinase results in significant reduction in M-CSF production (Table 1). These findings suggest that these two signaling molecules (e.g. ROK and PI3 kinase) are functionally linked to HA/CD44-mediated M-CSF production during breast tumor progression. We have also demonstrated that overexpression of ROK-RB (by transfecting MDA-MB-231 cells with ROK-RBcDNA) exhibits a dominant-negative effect on both p110 α and p110 β forms of PI3 kinase (but not the p110 γ or p110 δ form of PI3 kinase) (Fig. 8B) leading to an inhibition of HA-mediated M-CSF production in MDA-MB-231 cells (Table 1). These findings suggest that both p110 α and p110 β forms of PI3 kinase (to a lesser extent the p110 γ or p110 δ form of PI3 kinase) are most likely involved in the regulation of HA-mediated M-CSF production. Of course, we can not rule out the possibility that other PI3 kinase (e.g. p110 γ or p110 δ) may also be involved in M-CSF production in a HA-independent manner. The use of small interference RNA (siRNA) to selectively knockdown PI3 kinase (e.g. p110 α , p110 β , p110 γ or p110 δ form) and thereby interfere with PI3 kinase isoform-mediated functions and breast tumor progression is currently being investigated in our laboratory.

Finally, we have found that treatment of MDA-MB-231 cells with the PI3 kinase inhibitor (LY294002) (Table 2) or overexpression of the Rho-binding (RB) domain of ROK (by transfecting MDA-MB-231 cells with RBcDNA) (Table 2) induces reversal of tumor cell-specific phenotypes such as tumor cell growth and invasion (Table 2). Together, these findings strongly suggest that HA/CD44-mediated ROK activation and PI3 kinase signaling are closely coupled during the onset of tumor-specific behavior in MDA-MB-231 cells.

As summarized in Fig. 10, we propose that CD44 is tightly coupled with RhoGEF in a complex which can up-regulate RhoA signaling and Rho-Kinase (ROK) activity (Step 1). Activated ROK then phosphorylates certain cellular proteins including the linker molecule, Gab-1 (Step 2). Most importantly, phosphorylation of Gab-1 by ROK promotes the membrane localization of Gab-1 and PI3 kinase to CD44 and activates certain isoforms of PI3 kinase to convert PtdIns (4,5)P₂ to PtdIns (3,4,5)P₃ (Step 3) leading to AKT activation, cytokine (M-CSF) production and tumor cell behaviors (e.g. tumor cell growth, survival and invasion) required for breast tumor progression.

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(11) APPENDICES: (see attached Tables and Figures).

(12) BINDING: We have prepared the report according to the instruction provided by DOD.

(13) FINAL REPORTS:

Paper Publications:

1. Kalish, E., N. Iida, F.L. Moffat and Lilly Y. W. Bourguignon. A New CD44v3-Containing Isoform Is Involved in Tumor Cell Migration and Human Breast Cancer Progression. *Front Biosci.* 4:1-8 (1999).
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APPENDICES

FIGURE LEGENDS

Fig. 1: Detection of CD44v3-p115RhoGEF complex in MDA-MB-231 cells.

MDA-MB-231 cells were solubilized by 1% Nonidet P-40 (NP-40) buffer followed by immunoprecipitation and/or immunoblot by anti-CD44v3 antibody or anti-p115RhoGEF antibody, respectively as described in the Materials and Methods. Lane 1: Immunoblot of MDA-MB-231 cells with anti-CD44v3 antibody; Lane 2: Immunoblot of MDA-MB-231 cells with anti-p115RhoGEF antibody; Lane 3: Detection of CD44v3 in the complex by anti-p115RhoGEF-immunoprecipitation followed by immunoblotting with anti-CD44v3 antibody; Lane 4: Detection of p115RhoGEF in the complex by anti-CD44v3-mediated immunoprecipitation followed by immunoblotting with anti-p115RhoGEF antibody; Lane 5: Detection of anti-CD44v3-mediated immunoprecipitated materials blotted by an anti-p115RhoGEF-free serum (anti-p115RhoGEF antibody pre-absorbed by an excess amount of p115RhoGEF); Lane 6: Detection of anti-p115RhoGEF-mediated immunoprecipitated materials blotted by an anti-CD44v3-free serum (anti-CD44v3 antibody pre-absorbed by an excess amount of CD44v3).

Fig. 2: p115RhoGEF-mediated GDP/GTP exchange for RhoA protein.

P115RhoGEF isolated from MDA-MB-231 cells (treated with HA or without HA treatment) was preincubated with [³⁵S]GTPγS and GTPγS (or in the presence of 1mM unlabeled GTPγS) followed by adding GDP-loaded GST-RhoAGTPases (or GST alone). The amount of [³⁵S]GTPγS bound to samples in the absence of GTPases was subtracted from the original values. Data represent an average of triplicates from 3-5 experiments. The standard deviation was less than 5%. a: Kinetics of [³⁵S]GTPγS bound to GDP-loaded GST-tagged RhoA in the presence of p115RhoGEF (isolated from MDA-MB-231 cells treated with HA); b: Kinetics of [³⁵S]GTPγS bound to GDP-loaded GST-tagged RhoA in the presence of p115RhoGEF (isolated from MDA-MB-231 cells without HA treatment); c: Kinetics of [³⁵S]GTPγS bound to GDP-treated GST in the presence of p115RhoGEF (isolated from MDA-MB-231 cells treated with HA); d: Kinetics of [³⁵S]GTPγS bound to GDP-treated GST in the presence of p115RhoGEF (isolated from MDA-MB-231 cells without HA treatment).

Fig. 3: Detection of Rho-Kinase (ROK) in MDA-MB-231 cells.

Lane 1: Immunoblot of MDA-MB-231 cells with preimmune rabbit IgG; Lane 2: Immunoblot of MDA-MB-231 cells with rabbit anti-ROK IgG.

Fig. 4: Detection of Gab-1 phosphorylation by ROK *in vitro* (A) and the binding of ROK phosphorylated Gab-1 to CD44 (B).

A: The kinase reaction was carried out in the reaction mixture containing purified ROK, Gab-1 (obtained from anti-Gab-1-associated beads) and ATP in the presence of GTP γ S•GST-RhoA or GST-RhoA as described in the Materials and Methods.

a: Anti-phospho-serine-mediated immunoblot of Gab-1 (obtained from anti-Gab-1-associated beads) incubated with the reaction mixture in the absence of ROK (lane 1) or in the presence of ROK plus unactivated RhoA (RhoA without GTP γ S bound) (lane 2) or ROK plus activated RhoA [RhoA with GTP γ S bound (GTP γ S•RhoA)] (lane 3).

b: Anti-phospho-threonine-mediated immunoblot of Gab-1 (obtained from anti-Gab-1-associated beads) incubated with the reaction mixture in the absence of ROK (lane 1) or in the presence of ROK plus unactivated RhoA (e.g. RhoA without GTP γ S bound) (lane 2) or ROK plus activated RhoA [RhoA with GTP γ S bound (GTP γ S•RhoA)] (lane 3).

c: Anti-Gab-1-mediated immunoblot of Gab-1 (obtained from anti-Gab-1-associated beads) incubated with the reaction mixture in the absence of ROK (lane 1) or in the presence of ROK plus unactivated RhoA (e.g. RhoA without GTP γ S bound) (lane 2) or ROK plus activated RhoA [RhoA with GTP γ S bound (GTP γ S•RhoA)] (lane 3).

B: ¹²⁵I-labeled CD44cyt (the cytoplasmic domain of CD44 fusion protein) was incubated with Sepharose-beads containing Gab-1 phosphorylated by ROK plus activated RhoA [RhoA with GTP γ S bound (GTP γ S•RhoA)] (a) or ROK plus unactivated RhoA (e.g. RhoA without GTP γ S bound) (b) in the presence of various concentrations of unlabeled CD44cyt ranging from 10⁻¹² to 10⁻⁶M. The radioactivity associated with Gab-1-beads was analyzed by liquid scintillation counting as described in the Materials and Methods.

Fig. 5: Analyses of HA-induced *in vivo* phosphorylation of Gab-1 in ROK-RBcDNA (or vector)-transfected cells.

MDA-MB-231 cells (transfected with GFP-tagged ROK-PHcDNA or vector alone) were treated with HA (or no HA or pre-treated with anti-CD44 plus HA). These transfectants were then solubilized by NP-40, and immunoblotted with anti-GFP/anti-ROK (A) or immunoprecipitated with anti-Gab-1 antibody followed by immunoblotting with anti-phospho-serine or anti-phospho-threonine antibody (B).

A-a: Detection of ROK-RB fragment by anti-GFP-mediated immunoblot in cells transfected with vector alone (lane 1-3) or GFP-tagged ROK-RBcDNA (lane 4-6) [treated with no HA (lane 1 and 4) or with HA (lane 2 and lane 5) or pre-treated with anti-CD44 followed by HA (lane 3 and lane 6)].

A-b: Detection of endogenous ROK by anti-ROK-mediated immunoblot in cells transfected with

vector alone (lane 1-3) or GFP-tagged ROK-RBcDNA (lane 4-6) [treated with no HA (lane 1 and 4) or with HA (lane 2 and lane 5) or pre-treated with anti-CD44 followed by HA (lane 3 and lane 6)].

B-a: Anti-phospho-serine-mediated immunoblot of anti-Gab-1-mediated immunoprecipitated materials isolated from cells transfected with vector alone (lane 1-3) or GFP-tagged ROK-RBcDNA (lane 4-6) [treated with no HA (lane 1 and 4) or with HA (lane 2 and lane 5) or pre-treated with anti-CD44 followed by HA (lane 3 and lane 6)].

B-b: Anti-phospho-threonine-mediated immunoblot of anti-Gab-1-mediated immunoprecipitated materials isolated from cells transfected with vector alone (lane 1-3) or GFP-tagged ROK-RBcDNA (lane 4-6) [treated with no HA (lane 1 and 4) or with HA (lane 2 and lane 5) or pre-treated with anti-CD44 followed by HA (lane 3 and lane 6)].

B-c: Anti-Gab-1-mediated immunoblot of anti-Gab-1-mediated immunoprecipitated materials isolated from cells transfected with vector alone (lane 1-3) or GFP-tagged ROK-RBcDNA (lane 4-6) [treated with no HA (lane 1 and 4) or with HA (lane 2 and lane 5) or pre-treated with anti-CD44 followed by HA (lane 3 and lane 6)].

Fig. 6: Analysis of Gab-1-PI3 kinase complexes in MDA-MB-231 cells.

MDA-MB-231 cells transfected with ROK-RBcDNA (lane 3-4) or vector alone (lane 1-2) [either treated with 50µg/ml HA for 5 min (lane 2 or lane 4) or without any HA treatment (lane 1 or lane 3)] were solubilized and immunoprecipitated by anti-Gab-1 antibody followed by immunoblotting with mouse anti-p110α (a), anti-p110β (b), anti-p110γ (c), anti-p110δ (d) and anti-Gab-1 (e), respectively as described in the Materials and Methods.

Fig. 7: Interaction between the cytoplasmic domain of CD44 (CD44cyt) and Gab-1-PH fragment *in vitro* (A); and detection of CD44v3-Gab-1/PI3 kinase complex in Gab-1-PHcDNA-transfected/vector-transfected cells (B).

A-a: Illustration of Gab-1 full-length and Gab-1-PH fragmentcDNA construct. The full length Gab-1 contains pleckstrin homology (PH) domain (aa6-aa115) and the Met-binding domain (MBD-a proline-rich domain) (aa450-aa531). The Gab-1-PH fragment construct encodes the sequence between aa5-aa117.

A-b: Characterization of various recombinant proteins (FLAG-CD44cyt and Gab-1-PH-V5/His-bound beads) used in the *in vitro* binding assay.

Lane 1: Anti-FLAG-mediated immunoblot of FLAG-CD44cyt associated with V5/His-bound beads;
Lane 2: Anti-FLAG-mediated immunoblot of FLAG-CD44cyt associated with Gab-1-PH-V5/His-bound beads.

B: Analyses of CD44v3-Gab-1/PI3 kinase complex formation in MDA-MB-231 transfectants: MDA-MB-231 cells (transfected with Gab-1-PHcDNA or vector alone) treated with HA or no HA were

solubilized by 1% Nonidet P-40 (NP-40) buffer. Cell lysates were then used for anti-CD44v3-mediated immunoprecipitation followed by immunblotting with anti-CD44v3 antibody or anti-Gab-1 or anti-PI3 kinase antibody, respectively as described in the Materials and Methods.

B-a: Anti-p110 α -mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PHcDNA (lane 3 or lane 4) or vector alone (lane 1 and lane 2) treated with HA (lane 2 and lane 4) or no HA (lane 1 and lane 3).

B-b: Anti-p110 β -mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PHcDNA (lane 3 or lane 4) or vector alone (lane 1 and lane 2) treated with HA (lane 2 and lane 4) or no HA (lane 1 and lane 3).

B-c: Anti-p110 γ -mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PHcDNA (lane 3 or lane 4) or vector alone (lane 1 and lane 2) treated with HA (lane 2 and lane 4) or no HA (lane 1 and lane 3).

B-d: Anti-p110 δ -mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PHcDNA (lane 3 or lane 4) or vector alone (lane 1 and lane 2) treated with HA (lane 2 and lane 4) or no HA (lane 1 and lane 3).

B-e: Anti-Gab-1-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PHcDNA (lane 3 or lane 4) or vector alone (lane 1 and lane 2) treated with HA (lane 2 and lane 4) or no HA (lane 1 and lane 3).

B-f: Anti-His-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PHcDNA (lane 3 or lane 4) or vector alone (lane 1 and lane 2) treated with HA (lane 2 and lane 4) or no HA (lane 1 and lane 3).

B-g: Anti-CD44v3-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PHcDNA (lane 3 or lane 4) or vector alone (lane 1 and lane 2) treated with HA (lane 2 and lane 4) or no HA (lane 1 and lane 3).

Fig. 8: Detection of CD44v3-Gab-1/PI3 kinase complex in ROK-RB-transfected/vector-transfected cells (A) and measurement of PI3 kinase activity (B).

A: Analyses of CD44v3-Gab-1/PI3 kinase complex formation in MDA-MB-231 transfectants: MDA-MB-231 cells (transfected with ROK-RBcDNA or vector alone) treated with HA or no HA were solubilized by 1% Nonidet P-40 (NP-40) buffer. Cell lysates were then used for anti-CD44v3-mediated immunoprecipitation followed by immunblotting with anti-CD44v3 antibody or anti-Gab-1 or anti-PI3 kinase antibody, respectively as described in the Materials and Methods.

A-a: Anti-p110 α -mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with ROK-RBcDNA (lane 3 or lane 4) or vector alone

(lane 1 and lane 2) treated with HA (lane 2 and lane 4) or no HA (lane 1 and lane 3).

A-b: Anti-p110 β -mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with ROK-RBcDNA (lane 3 or lane 4) or vector alone (lane 1 and lane 2) treated with HA (lane 2 and lane 4) or no HA (lane 1 and lane 3).

A-c: Anti-p110 γ -mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with ROK-RBcDNA (lane 3 or lane 4) or vector alone (lane 1 and lane 2) treated with HA (lane 2 and lane 4) or no HA (lane 1 and lane 3).

A-d: Anti-p110 δ -mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with ROK-RBcDNA (lane 3 or lane 4) or vector alone (lane 1 and lane 2) treated with HA (lane 2 and lane 4) or no HA (lane 1 and lane 3).

A-e: Anti-Gab-1-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with ROK-RBcDNA (lane 3 or lane 4) or vector alone (lane 1 and lane 2) treated with HA (lane 2 and lane 4) or no HA (lane 1 and lane 3).

A-f: Anti-CD44v3-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with ROK-RBcDNA (lane 3 or lane 4) or vector alone (lane 1 and lane 2) treated with HA (lane 2 and lane 4) or no HA (lane 1 and lane 3).

B: Measurement of PI3 kinase activity: MDA-MB-231 cells (transfected with ROK-RBcDNA or vector alone) treated with HA or no HA. Membranes of these transfectants were used for anti-PI3 kinase-mediated immunoprecipitation. Precipitated proteins were incubated with phosphatidylinositol (4,5)P₂ and [γ -³²P]ATP, followed by extraction of lipids and separation by thin layer chromatography (TLC). The TLC plate was then exposed on an X-ray film. [PIP₃, phosphatidylinositol (3,4,5)P₂].

B-a: PI3 kinase activity detected by anti-p110 α -mediated immunoprecipitated materials isolated from membrane fraction of MDA-MB-231 cells transfected with ROK-RBcDNA (lane 3 or lane 4) or vector alone (lane 1 and lane 2) treated with HA (lane 2 and lane 4) or no HA (lane 1 and lane 3).

B-b: PI3 kinase activity detected by anti-p110 β -materials isolated from membrane fraction of MDA-MB-231 cells transfected with ROK-RBcDNA (lane 3 or lane 4) or vector alone (lane 1 and lane 2) treated with HA (lane 2 and lane 4) or no HA (lane 1 and lane 3).

B-c: PI3 kinase activity detected by anti-anti-p110 γ -materials isolated from membrane fraction of MDA-MB-231 cells transfected with ROK-RBcDNA (lane 3 or lane 4) or vector alone (lane 1 and lane 2) treated with HA (lane 2 and lane 4) or no HA (lane 1 and lane 3).

B-d: PI3 kinase activity detected by anti-p110 δ -materials isolated from membrane fraction of MDA-MB-231 cells transfected with ROK-RBcDNA (lane 3 or lane 4) or vector alone (lane 1 and lane 2) treated with HA (lane 2 and lane 4) or no HA (lane 1 and lane 3).

Fig. 9: Analyses of HA-induced *in vivo* phosphorylation of AKT-1 in ROK-RBcDNA (or vector)-transfected cells.

MDA-MB-231 cells (transfected with ROK-PHcDNA or vector alone) were treated with LY294002 (a PI3 kinase inhibitor) in the presence or absence of HA. These transfectants were then solubilized by NP-40, and immunoblotted with anti-phospho-AKT-1 (a) or rebotted with anti-AKT-1 (b).

a: Anti-phospho-AKT-1-mediated immunoblot of MDA-MB-231 cells transfected with vector alone (lane 1-4) or ROK-RBcDNA (lane 5 or lane 6) treated with HA (lanes 2, 4 and 6) or no HA (lanes 1,3 and 5) in the presence (lane 4 and lane 6) or the absence of LY294002 (lanes 1,2,3 and 5).

b: Anti-AKT-1-mediated immunoblot of MDA-MB-231 cells transfected with vector alone (lane 1-4) or ROK-RBcDNA (lane 5 and lane 6) treated with HA (lanes 2, 4 and 6) or no HA (lanes 1,3 and 5) in the presence (lane 3 and lane 4) or the absence of LY294002 (lanes 1,2,5 and 6).

Fig. 10: A proposed model for the interaction between CD44v3-mediated RhoA-ROK activation and Gab-1-linked PI3 kinase signaling during breast tumor progression.

HA-CD44 interaction is tightly coupled with p115RhoGEF in a complex which can up-regulate RhoA signaling and Rho-Kinase (ROK) activity (Step 1). Activated ROK then phosphorylates certain cellular proteins including the linker molecule, Gab-1 (Step 2). Most importantly, phosphorylation of Gab-1 by ROK promotes the membrane localization of Gab-1 and PI3 kinase to CD44 and activates certain isoforms of PI3 kinase to convert PtdIns (4,5)P₂ to PtdIns (3,4,5)P₃ (Step 3) leading to AKT activation, cytokine (M-CSF) production and tumor cell behaviors (e.g. tumor cell growth, survival and invasion) required for breast tumor progression.

Table 1: Measurement of M-CSF Production in the Media of MDA-MB-231 Cells and Transfectants.

A: Measurement of M-CSF production in untransfected cells:

<i>Treatments</i>	<i>Amount of M-CSF Production (pg/ml)</i>	
	No HA Addition (% of control)	HA Addition (% of control)
No IgG treatment (control)	75 ± 1.5 (100%)	198 ± 7.9 (264%)
Normal Rat IgG	77 ± 2.3 (102%)	199 ± 6.0 (259%)
Rat anti-CD44 IgG	74 ± 2.2 (99%)	73 ± 1.5 (98%)

B: Measurement of M-CSF production in untransfected cells treated with a PI3 Kinase inhibitor (LY294002):

<i>Treatments</i>	<i>Amount of M-CSF Production (pg/ml)</i>	
	No HA addition (% of control)	HA Addition (% of control)
No drug treatment (control)	74 ± 2.2 (100%)	199 ± 8.0 (268%)
LY294002 treatment	55 ± 1.0 (74%)	60 ± 1.2 (82%)

C: Measurement of M-CSF production in vector and ROK's RB-transfected cells:

<i>Transfectants Cells</i>	<i>Amount of M-CSF Production (pg/ml)</i>	
	No HA Treatment (% of control)	HA Treatment (% of control)
Vector-transfected (control)	75 ± 1.4 (100%)	195 ± 7.8 (260%)
ROK-RBcdNA-transfected	61 ± 0.6 (81%)	75 ± 1.3 (123%)

*MDA-MB-231 cells [untransfected (treated with or without LY294002) or transfected with ROK-RBcdNA or vector alone] were washed three times with serum free (SF)-DMEM and incubated in 3 ml of serum free-DMEM containing various reagents [e.g. HA (50µg/ml) or anti-CD44 antibody plus HA (50µg/ml) or without HA treatment] for 24 hr at 37°C in a 5% CO₂ humidified chamber. Subsequently, M-CSF concentrations in the conditioned medium and cells were determined using the Quantikine M-CSF immunoassay (R & D Systems).

Table 2: Measurement of Tumor Cell Growth and Invasion.

A: Analysis of Tumor Cell Growth:

<i>Cells</i>	Tumor Cell Growth (% of Control)	
	No HA Addition	HA Addition
Untransfected cells (control)	100	270
LY294002-treated untransfected cells	79	82
Vector-transfected cells	98	269
ROK-RBcDNA-transfected cells	62	75

B: Analysis of Tumor Cell Invasion

<i>Cells</i>	Tumor Cell Invasion (% of Control)	
	No HA Addition	HA Addition
Untransfected cells (control)	100	255
LY294002-treated untransfected cells	80	83
Vector-transfected cells	95	250
ROK-RBcDNA-transfected cells	54	58

*Procedures for measuring tumor cell growth and invasion in MDA-MB-231 cells [untransfected (treated with or without LY294002) or transfected with ROK-RBcDNA or vector alone] in the presence or absence of HA were described in the Materials and Methods.

Each assay was set up in triplicate and repeated at least 3 times. The values expressed in this table represent an average of triplicate determinations of 3-5 experiments with a standard deviation less than $\pm 5\%$.

Fig. 1

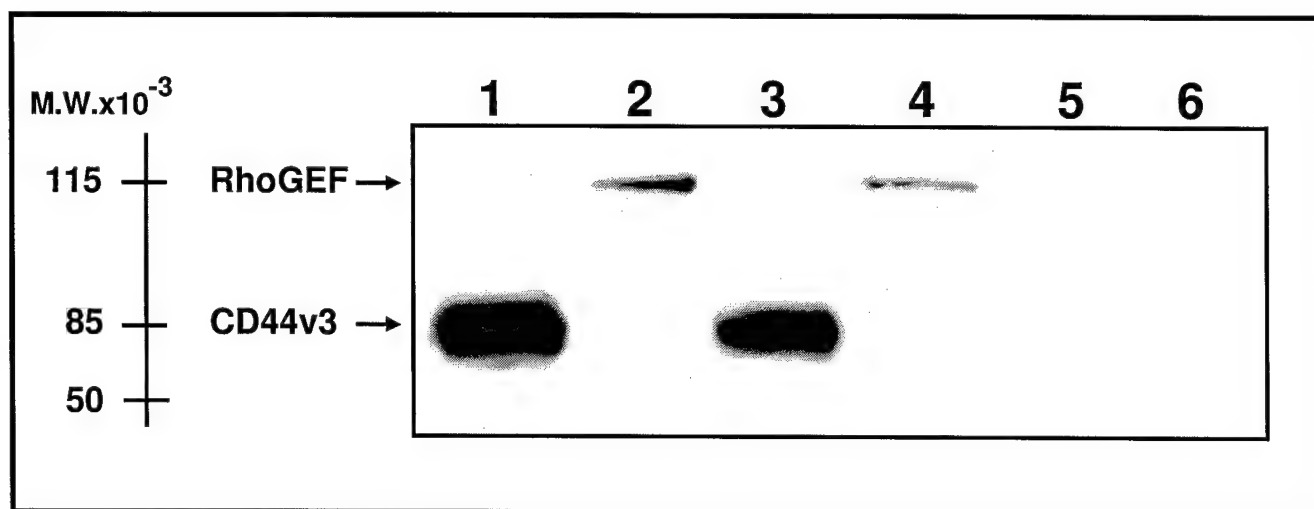


Fig. 2

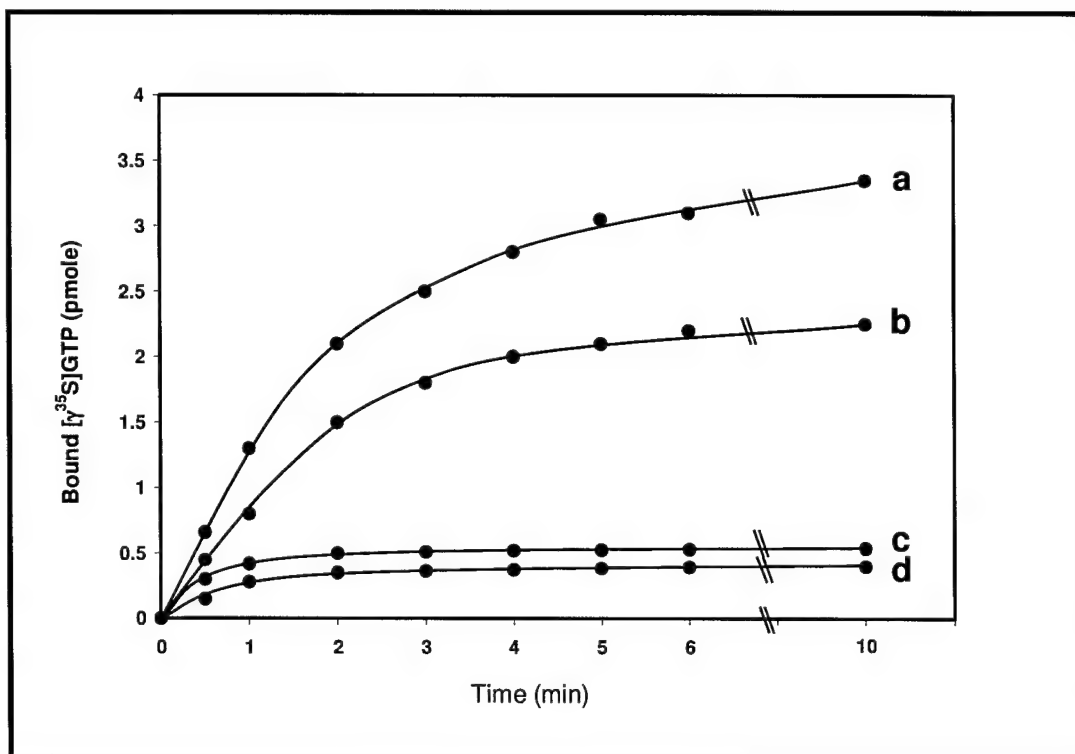


Fig. 3

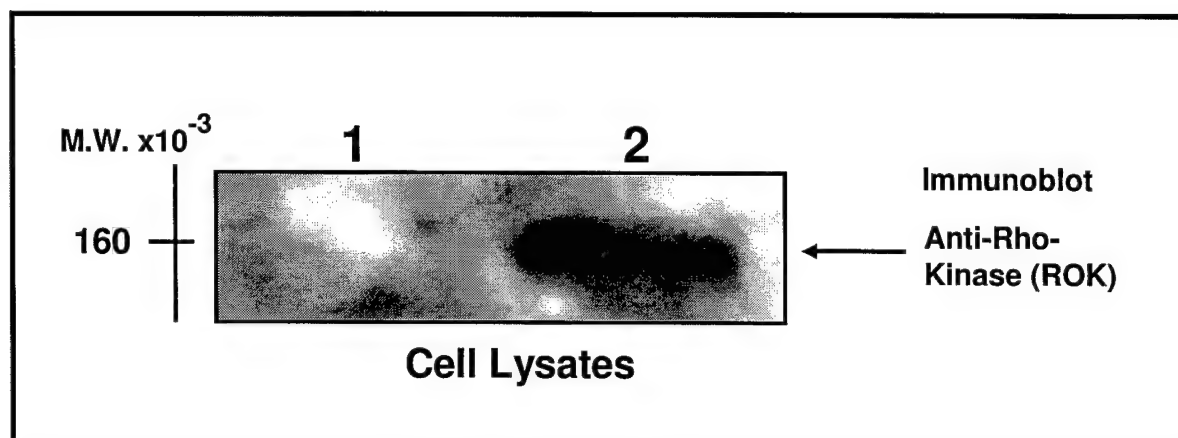


Fig. 4

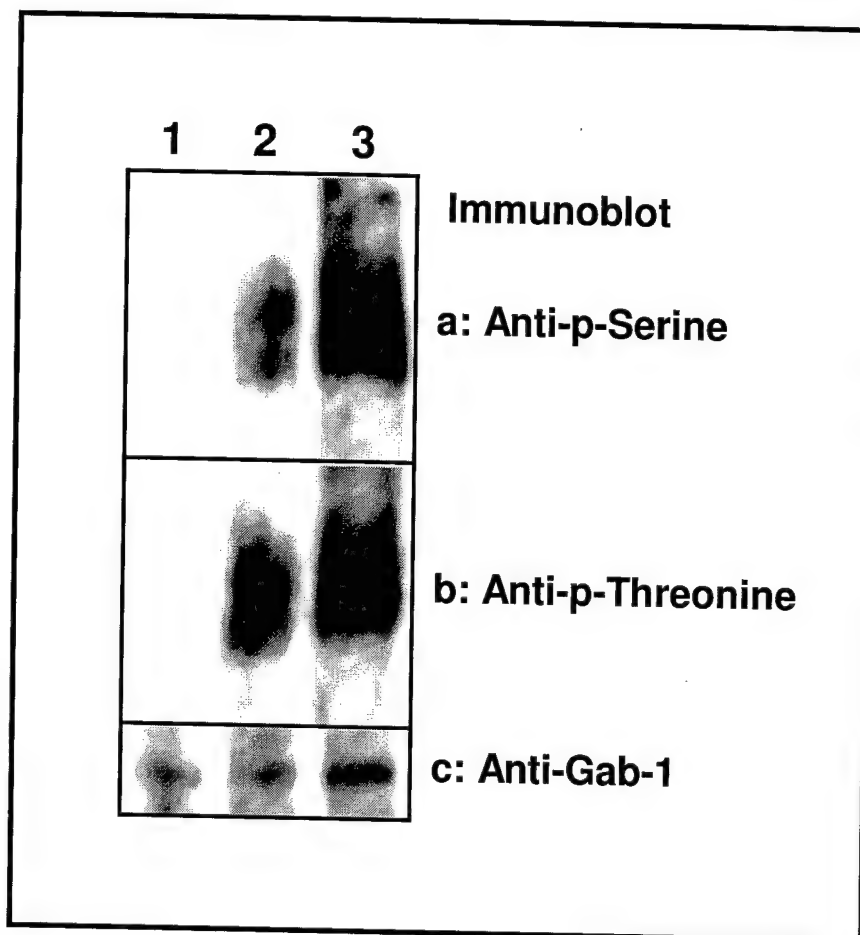


Fig. 5

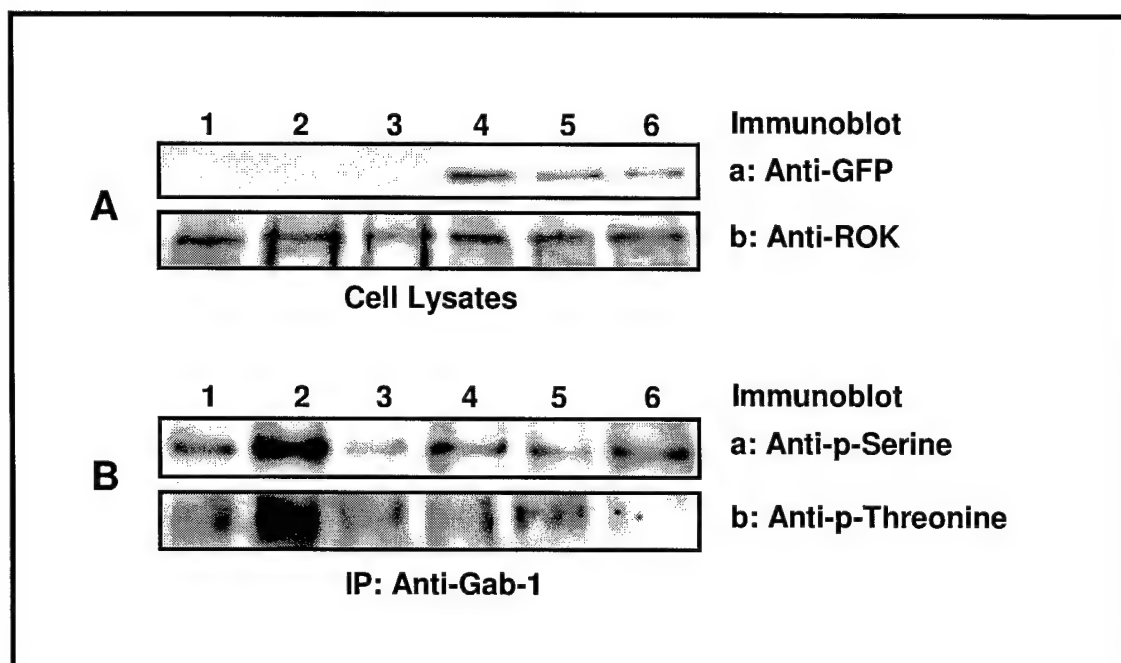


Fig.6

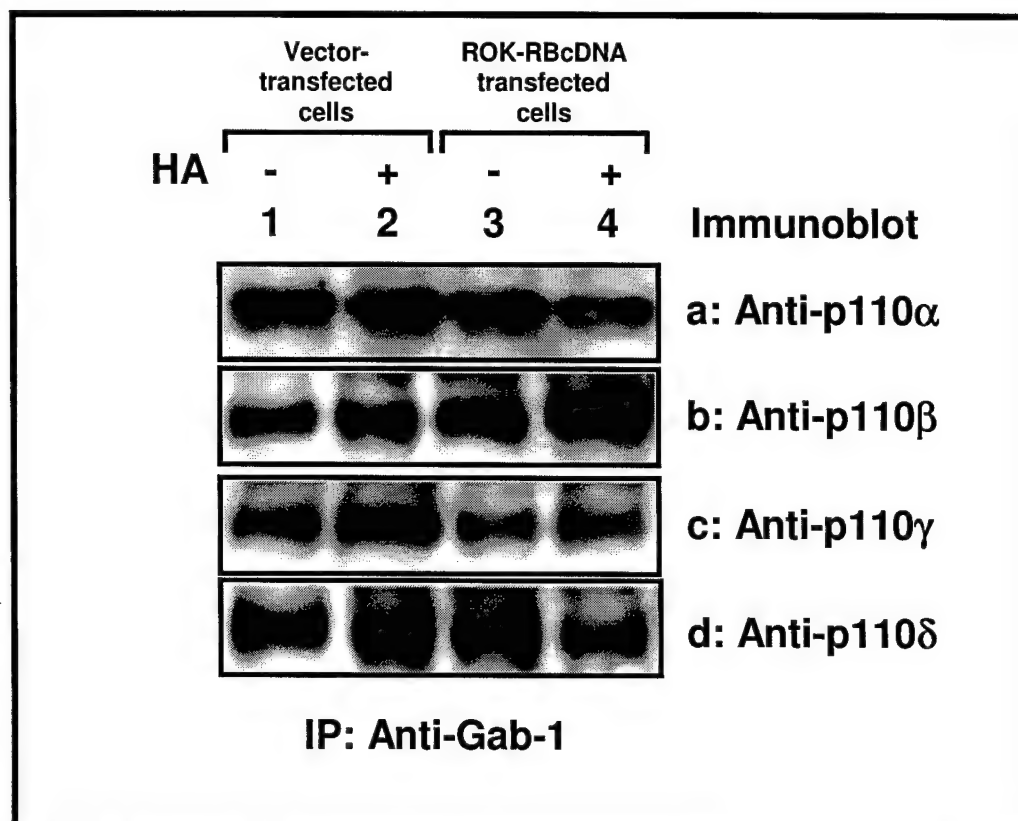


Fig.7A

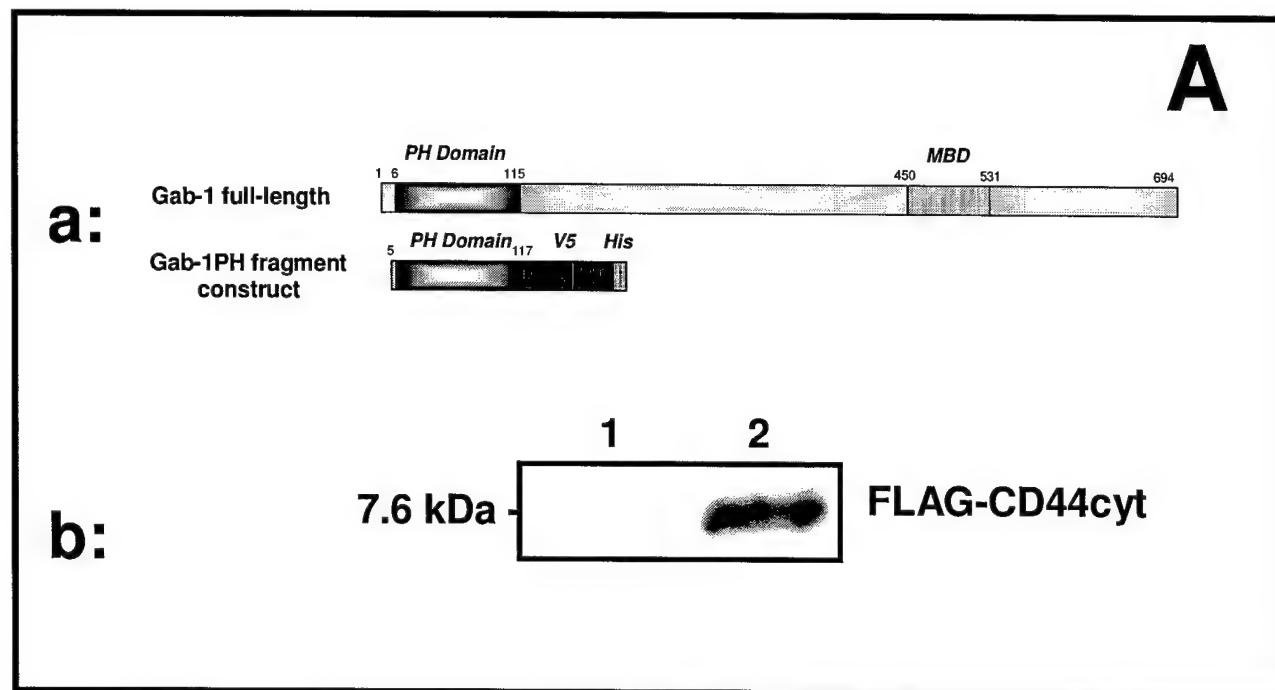


Fig.7B

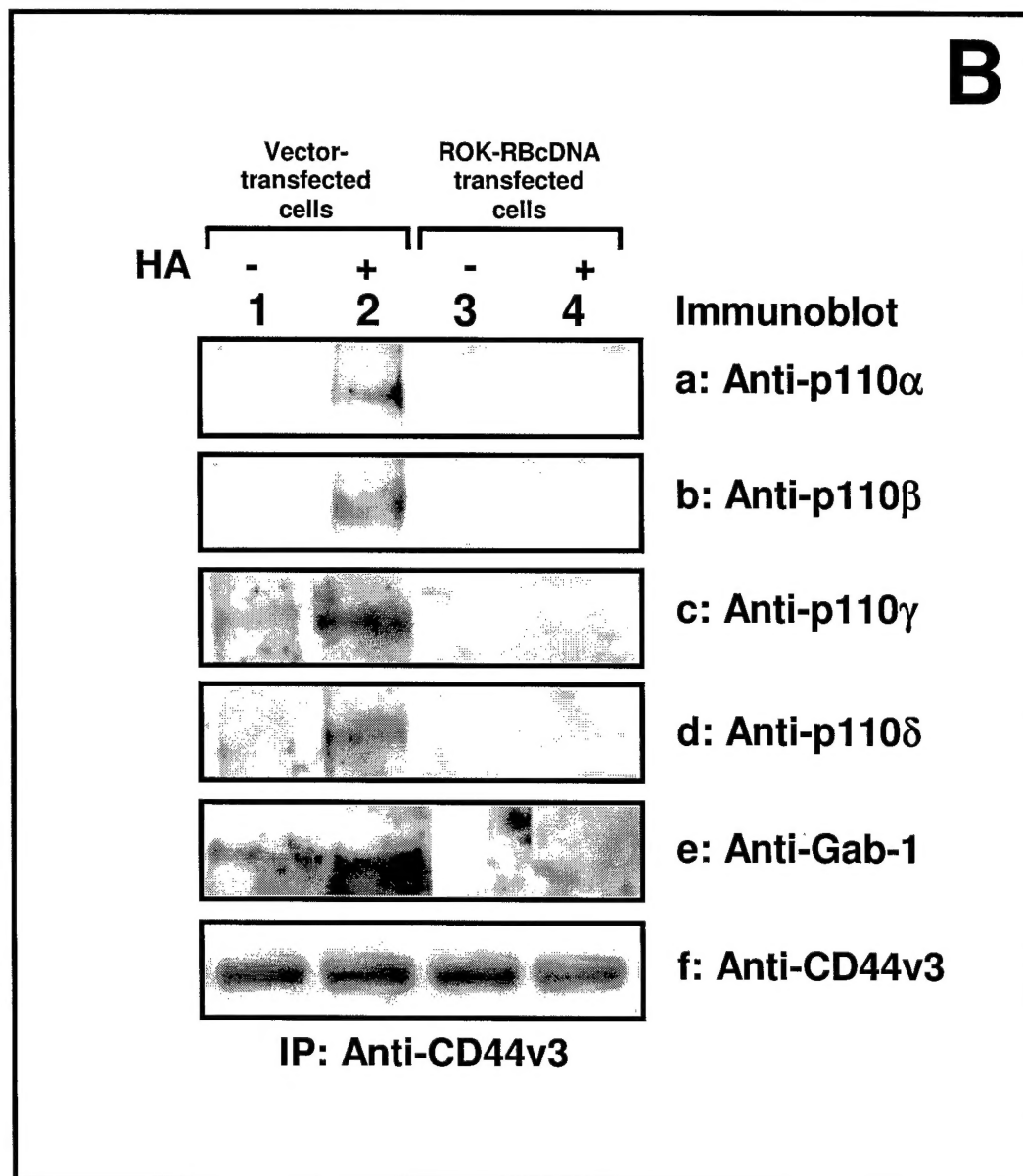


Fig.8

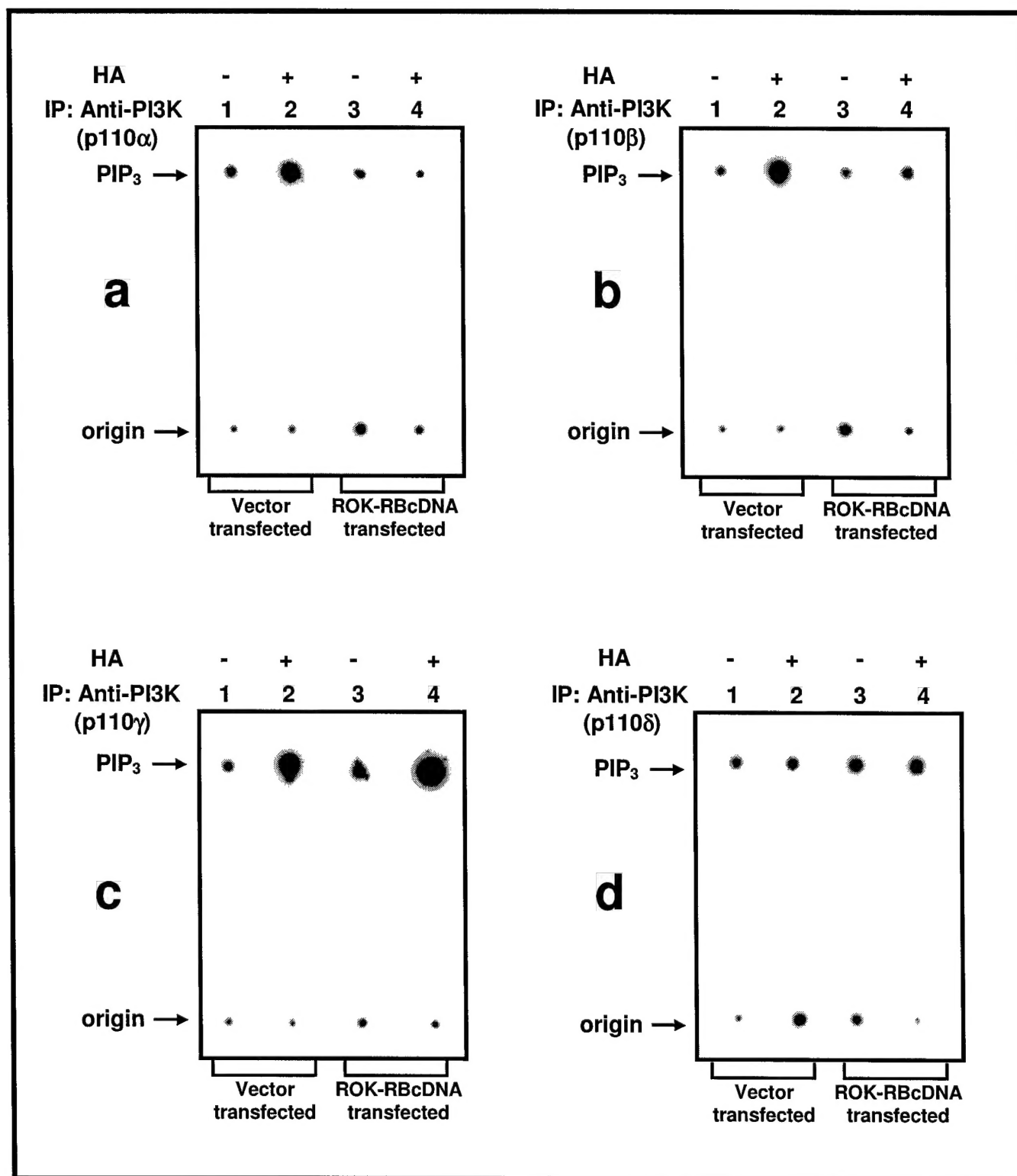


Fig.9

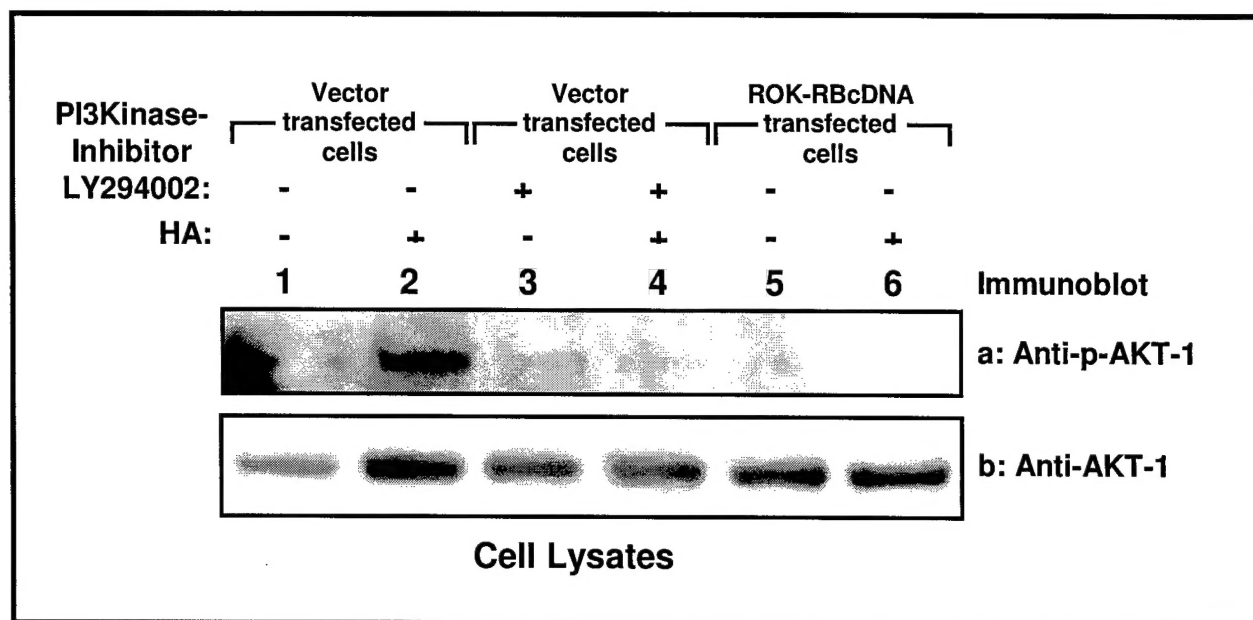


Fig.10

